Phylogenetic Analysis and Molecular Identification of Clawed Lobsters (Nephropidae) Based on Mitochondrial DNA

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Phylogenetic analysis and molecular identification of clawed lobsters (Nephropidae) based on mitochondrial DNA

by

HO Ka-chai

M. Phil. Thesis, Division of Biology

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Abstract

The first part of this thesis aims to elucidate the phylogenetic relationship of *Metanephrops* based on two mitochondrial gene regions, namely, large subunit rRNA (16S rRNA) and cytochrome *c* oxidase subunit I (COI) genes. Sixteen of the 17 extant species of this genus are included in this study. The results support the monophyly of the *binghami* group as well as *japonicus* group defined by morphology, but do not support the *arafurensis* and *thomsoni* groupings. The basal position of *M. challengeri* and *M. neptunus* in the phylogenetic trees provides evidence to support that *Metanephrops* evolved near Antarctica. Phylogenetic trees show that *japonicus* group is the most derived group among *Metanephrops* and species from this group are more related to some species in *thomsoni* group than to some species in *arafurensis* group. This result does not support that monophyletic origin of *arafurensis* group and does not support that *arafurensis* and *japonicus* groups are the oldest in *Metanephrops* as previously proposed. In addition, molecular data in this study are consistent with many relationships among species in this genus as suggested by morphology, e.g., the sister

relationship between *M. armatus* and *M. japonicus*. Molecular data as well as the morphological data from all species in *Metanephrops* are suggested to be combined together to elucidate a more robust phylogenetic relationship and evolutionary history of this genus.

The second part of this thesis aims to evaluate the potential of using two mitochondrial gene regions (16S rRNA and COI) as species identification tool in family Nephropidae. Two profiles have been set up using portion of 16S rRNA and COI gene sequences from 15 and 14 species, respectively. All species studied possess a unique sequence of both 16S rRNA and COI genes, except *Thaumastocheles dochmiodon* and *T. japonicus*. In the 16S profile, newly assigned test taxa can be discriminated successfully and are assigned to their corresponding major groups. The COI profile can also discriminate those newly assigned test taxa correctly. However, major groups of the test taxa could not be recovered in the COI profile. Comprehensive species identification profiles are suggested to set up for clawed lobsters in Nephropidae based on both 16S rRNA and COI genes.

用線粒體 DNA 研究螯蝦(海螯蝦科)的系統發育和物種鑒別

何家齊

香港中文大學生物學部碩士學位論文

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摘要

本論文的第一部分旨在透過分析在線粒體 16S 核糖體核糖核酸 (16S rRNA) 及細胞色素氧化酶 I 亞基(COI)基因的部分序列來闡明十六個屬於後海螯蝦屬(共十七個現存品種)的品種之間的系統發育。研究結果支持了以形態作出分類的 *binghami* 及 *japonicus* 組的單源性，但不支持 *arafurensis* 及 *thomsoni* 組的單源性。本研究中所建構的系統發育樹顯示 *Metanephrops challengeri* 和 *M. neptunus* 是在後海螯蝦屬各品種之間較原始的品種，這爲後海螯蝦屬是在位於近南極洲的地區進化出來的假設提供了證據。系統發育樹更顯示了 *japonicus* 組是較先進的組，相對一些屬 *arafurensis* 組的品種來說 *japonicus* 組與一些 *thomsoni* 組的螯蝦關係較接近。這並不支持 *arafurensis* 和 *japonicus* 組是較古老的組的假設。本研究中的分子數據結果部分支持了基於形態所確定的關係，例如：胄甲後海螯蝦 (*M. armatus*) 及日本後海螯蝦 (*M. japonicus*) 的姐妹群關係。

本論文的第二部分則旨在評估以 16S rRNA 及 COI 基因序列作爲海螯蝦科中的各品種物種鑒別的分子工具的可能。分別建立了擁有十五和十四個品種的 16S rRNA 及 COI 部分基因序列的物種庫。除了斜齒鋸指螯蝦 (*Thaumastocheles dochmiodon*) 及日本鋸指螯蝦 (*T. japonicus*) 外，其它所有研究的品種都有不同的 16S rRNA 及 COI 基因序列。16S rRNA 的物種庫能正確地鑒別新加入的測試

單元到所屬的品種，而且也能正確地區別到所屬的三個主要組。本研究中的 COI 物種庫亦能正確地辨別新加入的測試單元到所屬的品種，但不能成功把三個主要組區別出來。本研究建議建立一個由 16S rRNA 及 COI 基因組成用以辨別後海螯蝦屬中的各品種的物種庫。

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Chapter 1

General Introduction

1.1 Molecular phylogeny of *Metanephrops*

Metanephrops is the most speciose genus in family Nephropidae. There are 17 extant species and three fossil species recorded. Sixteen of the extant species are deep-water dwellers and 11 of them distribute in the Indo-Malay region (Chan, 1997). The genus *Metanephrops* was erected by Jenkins (1972) and species in this genus were previously referred to as *Nephrops*. Jenkins (1972) based on morphological feature, divided species of *Metanephrops* into four groups, namely, '*arafurensis*', '*binghami*', '*japonicus*' and *'thomsoni*'. The groupings were generally accepted by various authors (Chan and Yu, 1987; Holthuis, 1991; Chan, 1997). The distribution of *japonicus* group in the Indo-West Pacific is wide. The *thomsoni* group distributes from the western Pacific to western Australia, while the *arafurensis* group can be found in the Philippine-Australian region. The *binghami* group is the only group that distributes in the western Atlantic from southeastern parts of North America to the east of South America. Based on morphological traits, Jenkins (1972) proposed the phylogenetic relationships of species in *Metanephrops*. The *arafurensis* and *japonicus* groups are the oldest groups compared with the other two (Jenkins, 1972). Species from *thomsoni* group were suggested to have evolved from *arafurensis* group. In addition, *binghami* group is related *japonicus* group. Jenkins (1972) also

proposed that *Metanephrops* originated in the Indo-West Pacific region and species in *binghami* group arrived at Atlantic Ocean before or during the Lower Miocene. Chan (1997) agreed with this and further confined the originated area to the Indo-Malay region. On the other hand, Feldmann and Tshudy (1989) suggested that the place where *Metanephrops* originated is at the southern higher attitude, near to or at Antarctic region.

Mitochondrial large subunit ribosomal RNA (16S rRNA) and cytochrome *c* oxidase subunit I (COI) genes have been widely used in elucidating crustacean phylogeny at the species level (see Harrison and Crespi, 1999; Tong *et al.*, 2000; Ptacek *et al.*, 2001; Chu *et al.*, 2003; Harrison, 2004; Lavery *et al.*, 2004). However, up to now, studies based on molecular data about evolutionary relationship of species in *Metanephrops* are limited. Tam and Kornfield (1998) noted that the phylogeny inferred from morphological data may not fully reflect the true evolutionary relationships of Nephropidae. Molecular and morphological data would complement each other in phylogenetic studies of this clawed lobster family.

The division of the four species groups of *Metanephrops*, phylogeny, taxonomy and evolutionary relationships of *Metanephrops* up to now is mainly inferred from

morphological traits (Jenkins, 1972; Feldmann and Tshudy, 1989). The first part of this thesis aims to elucidate the phylogenetic relationships of *Metanephrops*, to evaluate the taxonomic status of the four groups defined by morphology and to elucidate the evolutionary history of *Metanephrops* based on DNA sequence analysis of partial segments of two mitochondrial gene regions, 16S rRNA and COI genes.

1.2 Identification of Nephropidae using DNA barcodes

The binomial taxonomic system has been established by Linnaeus in 1753 and this system is the basis of all biological researches. Traditional practice in taxonomy is mainly based on morphological features which differ among different groups of organisms. This practice requires a large number of taxonomic experts in different groups of organisms. Nowadays, the decline of biodiversity in natural environment has drawn many concerns. Studies in biodiversity conservation become a major issue among biological researches nowadays. However, the lack of taxonomic expertise and the difficulty in accessing taxonomic literatures were major problems encountered during biodiversity researches (Minelli, 2003).

The use of DNA barcode in species identification is suggested to overcome

these drawbacks in traditional taxonomy (Baker *et al.*, 2003; Blaxter and Floyd, 2003; Hebert *et al.*, 2003a; Tautz *et al.*, 2003) or, at least, to be one of the parameters in species identification (Blaxter, 2003; Mallet and Willmott, 2003; Paquin and Hedin, 2004). Hebert *et al.* (2003a) demonstrated that COI gene is a good molecular marker in species identification in various taxonomic levels of animals and COI barcode is proposed to be the universal marker for species identification. Moreover, it has been suggested that taxonomic identification based on one single gene is unreliable (Mallet and Willmott, 2003). Several studies have evaluated the potential and feasibility of other gene regions in taxonomic identification, such as small subunit nuclear ribosomal RNA (18S rRNA) and mitochondrial 16S rRNA genes (Floyd *et al.*, 2002; Blaxter *et al.*, 2003; 2004; Vences *et al.*, 2005).

The second part of this thesis aims to provide preliminary data for testing the potential of using sequences of 16S rRNA and COI genes in species identification in family Nephropidae and to evaluate the ability of these two mitochondrial genes in discriminating species not belonged to Nephropidae using profile trees based on these two genes.

Chapter 2

Literature Review

2.1 Molecular phylogenetic studies of crustaceans

2.1.1 Molecular phylogeny and reasons of using molecular markers in phylogenetic studies

Phylogeny is the study of evolutionary history of organisms and their relationships with the processes of evolution (Maddison, 1996). Molecular phylogeny is a stream of study on the evolutionary relationships of organisms derived from the genetic information through biological macromolecules such as DNA and proteins. In contrast to phylogenetic reconstruction based on morphology, inferences of evolutionary relationships of organisms in molecular phylogeny are based on molecular data directly or indirectly (Avise, 2004).

Avise (2004) stated several advantages on the use of molecular markers in phylogenetic studies. Molecular data contain genetic information that passes along the lineage, from parents to their offspring. By analyzing molecular data along the lineage of the taxa, the relationship of the lineage can be investigated.

By analyzing and comparing different gene segments in the genome of

organisms, a high-resolution view of lineage history, and also a better insight into a finer scale and detailed structure of phylogeny can be obtained (Maddison, 1996). The evolutionary rates of different genes in the genome of organisms are different and have their own patterns. The evolutionary history of organisms at different systemic levels can be estimated by comparing specific gene region in the genome of organisms (Crandall *et al.*, 2000; Avise, 2004).

Homologous feature is a structure shared by a set of species that are having the same phylogenetic origin, and this structure is present in their common ancestor, while analogous feature of a set of species is a structure that is similar in function but is not present in their common ancestor (Ridley, 2004). During phylogenetic reconstruction, homologous features provide phylogenetically informative data. Therefore, to distinguish between homologous and analogous features is one of the main themes in estimating phylogeny. Molecular data have an advantage that they can be used as a universal tool to distinguish homologous and analogous morphological characters in organisms. Moreover, morphological, physiological and behavioral characters in organisms can be influenced by non-genetic factors (e.g. environmental changes) that are phylogenetically uninformative and may provide misleading phylogenetic inferences. However, molecular data can be treated as

independent evidence to morphological characters in phylogenetic reconstruction of organisms (Simon *et al.*, 1994).

Molecular data provide a common yardstick for measuring divergence among organisms (Avise, 2004). The major problem encountered in morphology-based phylogeny is that among different groups of organisms, it is difficult to find a morphological structure which can be compared directly. Molecular approaches, on the other hand, provide a common platform in elucidating the relationship between different groups of organisms by comparing the same gene segment in the genome.

2.1.2 Characteristics of animal mitochondrial genome

Mitochondrial DNA (mtDNA) in most animals is a closed-circular macromolecule (Avise, 2004). The size usually ranges from 14 to more than 30 kilobases (kb) among different species (Harrison, 1989). The gene organization of animal mtDNA is simple. It contains two ribosomal RNA (rRNA) genes (12S and 16S), 22 transfer RNA (tRNA) genes, 13 protein coding genes (two ATPases, three cytochrome oxidases, a cytochrome *b* and seven NADHs), and a control region that is responsible for initiating mtDNA replication and transcription (Harrison, 1989; Avise, 2004). Animal mtDNA genome, in addition, lacks introns and seldom

possesses large families of repetitive DNA and sizable spacer sequences (Wolstenholme, 1992; Avise, 2004). Apart from the simple gene organization, animal mtDNA is maternally inherited and haploid, and possesses limited numbers of recombination (Saccone *et al.*, 1999). These features also facilitate mtDNA to be used in investigating the evolutionary history of organisms, as well as providing important insights into population structures, geographic variations and zoogeographical studies (Harrison, 1989).

2.1.3 Examples of crustacean phylogenetic studies derived from mitochondrial DNA

The first crustacean mtDNA was obtained in a study on the genome organization in *Artemia* (Batuecas *et al.*, 1988). Four years later, the first crustacean phylogenetic study based on mtDNA was reported (Cunningham *et al.*, 1992). Phylogenetic relationships of two Alaskan genera of king crabs and nine genera of hermit crabs were inferred from mitochondrial large subunit ribosomal RNA (16S rRNA) gene sequences. The two king crab genera are placed within the hermit crab genus *Pagurus*. The conclusion based on 16S rRNA gene sequences was consistent with the conclusion based on previous morphological based taxonomy. 16S rRNA gene data further suggested the sister relationship between the two morphological similar hermit crab species, *Pagurus acadianus* and *P. bernhardus*, and this

relationship is also supported by the morphological features.

MtDNA were used to evaluate the traditional morphology-based taxonomy and phylogeny. The morphology-based taxonomic groupings among seven species in a shrimp genus *Metapenaeopsis* were evaluated by using partial sequences of 16S rRNA and cytochrome *c* oxidase subunit I (COI) genes (Tong *et al.*, 2000). Molecular data results supported the previously proposed taxonomic divisions as well as the hypothesis that the shape of petasma (male genitalia of shrimp) is phylogenetically highly significant in elucidating phylogenetic relationships among *Metapenaeopsis* species.

Four mtDNA genes, 16S rRNA, small subunit rRNA (12S rRNA), COI and cytochrome *b* (Cyt *b*), have been used to evaluate the phylogenetic relationships, and to compare the nucleotide variations within and among four freshwater crayfishes in genus *Cherax* (Munasinghe *et al.*, 2003). The results inferred from the former three gene sequences were consistent with each other and the results supported the taxonomic status and phylogenetic relationships of the four *Cherax* species deduced from previous study based on allozyme electrophoresis and morphological features. As the trees inferred from Cyt *b* sequences were found to be incongruent with the

other three gene regions and two divergent segments of Cyt *b* were amplified from a single crayfish specimen. Therefore, Munasinghe *et al.* (2003) suspected that Cyt b pseudogene in at least one of the species was amplified.

It is difficult to elucidate the phylogenetic relationships between morphologically similar taxa based only on morphological criteria. MtDNA is helpful in elucidating the relationships between these taxa. The basic color pattern on the abdomen of a Florida spiny lobster *Panulirus argus*, for example, is the only difference between Brazilian and Caribbean populations. Traditionally, body color is not used as a character for taxonomic classification. The taxonomy of *P. argus*, therefore has remained unchanged since it was originally described. Sarver *et al.* (1998) compared two populations of *P. argus* from these two regions based on 16S rRNA and COI gene sequences. Results showed that the level of sequence divergence between *P. argus* from these two regions may be high enough to suggest that the two populations would represent the two subspecies, namely *P. argus argus* and *P. argus westonii*.

MtDNA can be used to infer the phylogenetic relationship of morphologically similar crab species. The genus *Brachynotus* consists of four species, *B. foresti*, *B.*

gemmellari, *B. sexdentatus* and *B. atlanticus*. The former three species are mostly endemic to the Mediterranean while *B. atlanticus* distributes along the Atlantic coast of northern Africa, southern Europe and extends into the western Mediterranean (d'Udekem d'Acoz, 1999). *B. gemmellari* and *B. sexdentatus* can be distinguished by their bathymetric and morphometric differences (Froglia and Manning, 1978). 16S rRNA gene sequences analysis supported the monophyly of genus *Brachynotus*, in which *B. atlanticus* and *B. foresti* formed a distinct clade while all 16S rRNA gene sequences of *B. gemmellari* and *B. sexdentatus* are the same (Schubart *et al.*, 2001). The lack of sequence variation between *B. gemmellari* and *B. sexdentatus* leads Schubart *et al.* (2001) to suggest that there was a recent separation event or continuing gene flow between these two taxa. Based on the results, it was suggested that both taxa may represent two ecophenotypes of a single species. A review of the taxonomic status of these two species, therefore, is suggested. The findings from this study provide information not only for the present taxonomic classification of this genus of crabs, but also indicate that mtDNA is useful for understanding the evolutionary relationship of this crab genus.

Apart from aiding in distinguishing morphological similar taxa, mtDNA is also a powerful tool to evaluate current taxonomy of organisms based on morphology.

The taxonomy of grapsoid crabs (superfamily Grapsoidea) has remained unchanged since 1900 and the relationships among genera of Grapsidae were rarely questioned. The phylogeny of American grapsoid crabs was elucidated based on 16S rRNA gene data (Schubart *et al.*, 2000a). In addition, this study verified the present taxonomic relationships of grapsoid crab species. Results from this study were consistent with many aspects of current morphological systematics of grapsoid genera. In traditional grouping, there are six American grapsid genera (*Geograpsus*, *Goniopsis*, *Grapsus*, *Leptograpsus*, *Pachygrapsus* and *Planes*) included in subfamily Grapsinae. This group was strongly supported by the consensus tree inferred from 16S rRNA gene data. In addition, this Grapsinae group was also supported by previous systematic study based on larval morphologies (Cuesta and Schubart, 1999). 16S rRNA data suggested that two genera of Sesarminae, *Chasmagnathus* and *Cyclograpsus*, should be relocated to the subfamily Varuninae, and this was previously suggested in a morphological study on zoea (Schubart and Cuesta, 1998). Moreover, the relocation and reclassification of two Varuninae genera, *Glyptograpsus* and *Platychirograpsus*, as concluded from a previous larval morphological study is also evidently supported by the 16S rRNA gene data. Results also suggested that the status of *Percnon* within the subfamily Plagusiinae is controversial and needs further investigation. The molecular data from this study confirm many traditional taxonomic relationships

among superfamily Grapsoidea. In order to have a better resolution of phylogeny of this superfamily, more crab samples of genera from Indo-West Pacific should be included in further studies (Schubart *et al.*, 2000a).

In addition to evaluate the current taxonomic status of crustaceans, mtDNA also provides information to resolve the complicated relationships among and within genera. Based on morphological characteristics from exopod, spiny lobsters of *Panulirus* can be divided into four informal groups, Groups I to IV. In a molecular phylogenetic study of *Panulirus* (Ptacek *et al.*, 2001), mitochondrial 16S rRNA and COI genes sequences and the combined data set analyses supported the separation of Groups I/II and Groups III/IV. In this study, the relatively higher level of genetic divergence among species in Groups I and II suggested that these two groups radiated earlier than other groups. In contrast, the relatively lower level of genetic divergence among species in Groups III and IV suggested that there is a more recent radiation event of species in these two groups. Based on morphologies of modern *Panulirus* lobsters, George and Main (1967) suggested that *P. argus* and *P. longipes* (both belong to Group I) represent the ancestral species. All phylogenetic trees inferred from mitochondrial genes suggested that there was an early radiation event between *P. argus* and *P. interruptus* lineages, and then followed by a radiation of

remaining species in Group I. Allopatric speciation within Groups III and IV lineages has been hypothesized by George and Main (1967). Molecular data provided some support to this hypothesis. For example, based on maximum parsimony tree inferred from COI gene data set and maximum likelihood trees based on both 16S rRNA gene and combined data sets, *P. polyphagus* (a Group III species) is the first species to split off in Groups III/IV lineage. MtDNA sequences analyses in this study clarified many complicated phylogenetic relationships within genus *Panulirus*. Some relationships of the species within these groups still could not be resolved. It is necessary to undergo further investigation in order to understand the pattern of speciation and evolutionary history of this commercially important lobster genus.

The classification, evolutionary relationship and taxonomy of some commonly known crustacean species are still controversial and mtDNA was used to elucidate these matters. The genus *Penaeus sensu lato* (as defined by Fabricius, 1798), is an economically important, well-known and diverse group of marine shrimp. Several authors (e.g. Holthuis, 1980) treated the morphological-based division of *Penaeus s. l.* as six subgenera (*Farfantepenaeus, Fenneropenaeus, Litopenaeus, Marsupenaeus, Melicertus* and *Penaeus sensu stricto*), but this division was not always accepted by recent authors (Yu and Chan, 1986; Chan, 1998). A recent revision on the families

and genera of Penaeoidae raised all the six subgenera to genus level (Pérez Farfante and Kensley, 1997). Several studies are conducted to evaluate the validity of these groupings based on molecular data (Baldwin *et al.*, 1998; Gusmão *et al.*, 2000; Maggioni *et al.*, 2001). Due to the limitations and constraints in these studies, the relationships among and within species of *Penaeus* are still controversial. Lavery *et al.* (2004) conducted a comprehensive phylogenetic reconstruction of the *Penaeus* shrimp based on sequence analyses of 16S rRNA and COI genes. Molecular data only supported two natural groups in *Penaeus*, one group consists of the subgenera *Marsupenaeus* plus *Melicertus*, and the other group includes subgenera *Farfantepenaeus, Fenneropenaeus*, *Litopenaeus*, and *Penaeus s.s.* The six previously proposed subgenera divisions were not supported in Lavery *et al.* (2004). The results from this study supported that only two of the subgenera, *Farfantepenaeus* and *Litopenaeus* are monophyletic, but this classification was previously rejected (Baldwin *et al.*, 1998; Gusmão *et al.*, 2000). In addition, these two Atlantic groups of *Penaeus* are closely related in the phylogenetic trees, supporting that there was only one relatively recent colonization event from the western Pacific to America in *Penaeus s.l.* species. Lavery *et al.* (2004) concluded that more molecular data and especially from nuclear DNA, should be obtained before conclusion can be drawn to raise all subgenera to genera level, and to further confirm the relationships and status

between and among species in *Penaeus*.

In order to have a better understanding of phylogeny of organisms, morphological data were often combined with molecular data to infer the species relationships. Harrison and Crespi (1999), for example, conducted a study of the evolutionary history of the crab genus *Cancer* based on both adult morphological features and COI gene sequences. Phylogenies inferred from morphological characters and COI data independently indicated considerable difference in topology, but each of the data sets was strongly supportive of itself. COI data set, and the combined morphology and COI data sets were believed to infer a more accurate hypothesis on the phylogeny of this genus respectively. COI and the combined data sets suggested a closer relationship between two Atlantic species (*C. borealis* and *C. pagurus*) than to any other species from the Pacific. The suggestion based on molecular data was also supported by the paleontological evidence, that *Cancer* was originated in the North Pacific, then migrated toward south, and invaded to the Atlantic Ocean via the coast of North and South America. The timing and location of the origin of this genus based on fossil records were also consistent with the phylogeny inferred from maximum likelihood analysis of the COI data. The molecular clock generated from COI data in this study suggested that the invasion

event of *Cancer* crabs from North Pacific to Atlantic Ocean occurred at approximately 6-12 million years ago. Various studies, however, estimated several different divergence times between closely related Atlantic and North Pacific marine species, ranging from 1.7-8.9 million years ago (Grant *et al.*, 1984; Grant, 1986; Grant and Stahl, 1988). Due to several different divergence times of Atlantic and North Pacific marine species were proposed, Harrison and Crespi (1999) suggested further study should be carried out on the timing and dispersal patterns of various marine species between the Atlantic and Pacific Oceans.

2.2 Identification of species based on DNA barcode

2.2.1 Traditional taxonomy and its current practice

Taxonomy involves definition, description, classification and nomenclature of living organisms. As the basis of all biological researches, taxonomy not only provides foundation knowledge for phylogenetic studies (Wheeler, 2004), but also provides necessary species database and catalogue for ecology and conservation studies (Gotelli, 2004; Mace, 2004). Taxonomy, moreover, makes accessible the vast and most valuable benefits offered by biodiversity to the human society (Tautz *et al.*, 2003; Wilson, 2004).

The naming system in taxonomy we use today is a binomial system which was

first introduced by Carolus Linnaeus in 1753. The nomenclature of a species based on this system consists of two words, such as '*Homo sapien*'. Traditional taxonomy practice is mainly based on morphological traits, and is still being adapted nowadays. When an organism is discovered and suspected to be a new species, three steps will be done by taxonomists (Knapp *et al.*, 2004). First, when an organism is discovered, all relevant literatures will be checked and searched thoroughly by the taxonomist, to see whether or not establishing and describing a new species is necessary. Second, after the organism is confirmed to be a new species, its morphological characters and taxonomic status will be studied, described and defined in a literature, traditionally the literature will be published in paper medium. After the publication of the literature, the final step is that the described type specimen will be deposited in museum for future reference and comparison. This practice, however, is expert demanding, and the number of experts is declining in these years (Tautz *et al.*, 2003; Gaston and O'Neill, 2004).

2.2.2 Needs for DNA barcode

It is estimated that there are about 10 million species on Earth (Gotelli, 2004; Wilson, 2004) and this is a vast and most valuable benefit to the human society. The number of taxonomists on all living organisms nowadays is between 6000 and 10000

(Gewin, 2002; Wilson, 2004) and about 10% of the Earth's species were described only. Traditional taxonomy needs a large number of taxonomic experts for different groups of living organisms. There is a general decline of taxonomic studies and knowledge in recent years (Tautz *et al.*, 2003; Gotelli, 2004; May, 2004; Wheeler, 2004) which leads to the so called 'taxonomic impediments' in biodiversity and conservation studies recently (Hebert *et al.*, 2003a; 2003b; Tautz *et al.*, 2003). Biodiversity conservation has become a major social and economic issue due to the decline in biodiversity. In addition to the lack of taxonomic experts to identify and describe species, difficulty in accessing taxonomic literatures (Minelli, 2003) is another impediment during biodiversity researches. These factors may also create barriers to other biological researches. In addition, delicate specimens are easily damaged during collection in museums, in which characters may be lost for identification. Cryptic species may be neglected by traditional taxonomists because of their morphological similarity (Witt and Hebert, 2000; Hebert *et al.*, 2004a; Smith *et al.*, 2006). Genetic data, therefore, were suggested by various biologists to assist in overcoming these 'impediments' to complement traditional species identification (Floyd *et al.*, 2002; Baker *et al.*, 2003; Blaxter and Floyd, 2003; Hebert *et al.*, 2003a; Proudlove and Wood, 2003; Tautz *et al.*, 2003; Blaxter, 2004; Hebert and Gregory, 2005; Schander and Willassen, 2005). It has been proposed to use the mitochondrial

COI gene sequence as DNA barcode for species identification in the animal kingdom in overcoming these 'impediments' (Hebert *et al.*, 2003a).

DNA barcode for species identification can be served as a fast, accurate and automatable way in species identification (Hebert and Gregory, 2005). DNA barcodes from different organisms can be easily obtained from a standard protocol (Blaxter, 2004), and no prior knowledge on the genome of the organisms is needed. DNA barcodes of organisms can be determined by any life stages, any body parts, and even specimens without any diagnostic characters (Stoeckle, 2003; Schander and Willassen, 2005). Due to the ease of accessing and obtaining organism's DNA, scientists suggested that a unitary taxonomy database and gene sequence of organism to be included in the database on the web (Godfray, 2002; Wilson, 2003; Gotelli, 2004; Janzen, 2004). The duplicated description of species which often encountered in the present taxonomic practice can be prohibited, if such web-based system is utilized (Lee, 2002). Some scientists argue that barcode system cannot replace traditional morphology for species identification (Dunn, 2003; Lipscomb *et al.*, 2003; Seberg *et al.*, 2003; Moritz and Cicero, 2004; Will and Rubinoff, 2004; Will *et al.*, 2005). Various authors, however, have proposed that DNA barcode may, at least, be served as one of the criteria for taxonomic identification (Blaxter, 2003; Mallet and

Willmott, 2003; Paquin and Hedin, 2004). Indeed, taxonomic studies and combination of various types of biological information (e.g. morphology, behavior, genetics, etc.) is required before the setting up and implementation of such molecular identification system. The gathering of multiple types of biological information during the establishment of the DNA barcode system, can also facilitate the development of traditional taxonomy researches (Paquin and Hedin, 2004).

2.2.3 Molecular identification based on DNA barcodes

In order to demonstrate the feasibility of DNA barcode, Hebert *et al.* (2003a) used mitochondrial COI gene sequences for species identification in the animal kingdom. In this study, three COI profiles have been set up to evaluate the potential of COI barcode as an identification tool at different systematic levels. First, sequences of 100 species in seven animal phyla were used to construct a COI profile. From this COI phylum profile, there were only two misidentifications. Hebert *et al.* (2003a) explained that the two misidentifications were due to the limited sampling size and diversity in this study. Second, a profile has been set up for eight of the most diverse orders of insects with 100 families. This COI ordinal profile has succeeded in discriminating all insect families. Third, as Lepidoptera is one of the most speciose orders of insects and COI sequences divergence are low among families of

lepidopterans, another COI profile, was constructed to include 200 closely allied lepidopteran species in three superfamilies. The setting up of COI species profile was 100% successful in identifying lepidopteran species, that all lepidopteran species possess a distinct sequence. The success in discriminating different taxa at different systematic level in the three COI profiles respectively demonstrated that COI barcode could be served as a reliable identification system in various taxonomic levels from phyla to species in animal kingdom.

Although the ability of COI barcode for species identification in the animal kingdom was demonstrated in Hebert *et al.* (2003a), concern about the ability of COI barcode for identification among closely related taxa has been addressed (Mallet and Willmott, 2003). The ability of COI gene sequence in discriminating of closely related species has been demonstrated in all animal phyla (Hebert *et al.*, 2003b). In this study, there were more than 98% closely related species pairs with sequence divergence greater than 2%. Cindaria is an exceptional taxon due to the relatively low rate of evolution in its mitochondrial gene, which results from the excision repair system in these animals. The stasis in evolution of cnidarian mitochondrial genome contributed to the relatively low genetic divergence among cnidarian species. In addition, the relatively high level of COI divergence in the Fungi and Protista

suggested that COI barcode identification system can also be implemented to these kingdoms.

Springtail (order Collembola) is one of the most diverse groups of soil arthropods. In Canadian Artic, the distribution and taxonomy of springtail are poorly known. Hogg and Hebert (2004) examined the utility of COI barcode for species identification of Canadian artic Collembola. The results showed that 19 species in 13 genera of Collembola were discriminated successfully. COI gene sequence divergences between the species studied, were at least greater than 8%, whereas divergence within species were less than 1%. Two exceptions were discovered. The sequence divergence among individuals of *Sminthurides* and *Folsomia* were up to 5% and 13% respectively. Hogg and Hebert (2004) explained that these might be the presence of unknown sibling species. Further studies, therefore, are needed to reveal the cryptic diversity in these genus. This study demonstrates that COI barcode is an effective tool in species identification in Collembola. It also indicates the needs of a taxonomic identification system and taxonomic revision for this group of organisms.

DNA barcode is also effective in discriminating vertebrate animals. A preliminary bird identification system was set up for 260 species in North America

(Hebert *et al.*, 2004b). There were no bird species sharing the same COI barcode in this study, and the sequence divergence between two closely related species was 18 times higher than the divergence within the same species. Thus DNA barcode in bird identification from North America was feasible and effective. Yet in this study, only COI barcode of 40% North America birds have been sequenced. It is suggested that more barcodes is needed in setting up a comprehensive identification system for this one of the best-studied vertebrate groups.

DNA taxonomy can also assist in revealing the distribution and level of biodiversity of endangered species, as well as species that are difficult to collect. The traditional species identification in American endangered cave spider genus *Cicurina*, is mainly based on genital morphology, but adult spiders were very difficult to collect in the field. COI barcodes were used to identify immature cave spider, in order to reveal the species diversity and their distribution in America (Paquin and Hedin, 2004). Results showed that among 104 COI sequences, a large number of specimens could be fitted into distinct terminal clades in the phylogenetic tree. Those specimens at terminal clades corresponded to prior species described based on morphologies. As a result, it was successful to identify a number of immature spider specimens studied and to place species name on them. Three inconsistent cases between the COI

barcode results and prior species hypotheses were identified in this study. These inconsistency may be due to introgression events between *Cicurina* species or synonymous species name. Further studies are suggested to investigate their relationships. In addition, there were some genetic variations between *C. madla* from four different sites. Conservation measures are needed to protect this endangered species in these four regions respectively. Paquin and Hedin (2004) concluded that DNA barcode is a powerful tool in revealing species taxonomy, biodiversity and conservation issues. It can provide not only the evidence to identify previous unidentified species, but also provide information for species conservation, species diversity, distribution of species, species that are difficult to collect as well as endangered species. The authors further stated that it is not recommended to use taxonomic studies based on one parameter (e.g. morphology) only. In order to obtain more accurate results in species identification and taxonomic studies, combination of multiple types of biological information (e.g. morphological, physiological, ecological and genetic data) should be included in these studies.

Very small genetic divergence or identical COI barcodes would be resulted from closely related species (Harrison, 2004; Lorenz *et al.*, 2005). This can lead to inaccurate species identification. Various scientists also suggested that it is not

plausible to rely on a single gene region for species identification (Mallet and Willmott, 2003; Stoeckle; 2003; Schander and Willassen, 2005). Several studies were carried out to evaluate the ability of using other gene regions, such as ribosomal RNA (rRNA) genes, as DNA barcode for species identification. For example, Nematoda is one of the animal groups in which most of the species were undescribed (Schander and Willassen, 2005). Floyd *et al.* (2002) developed an identification system for soil nematodes using partial sequence of small subunit nuclear rRNA (18S rRNA) gene. They suggested that sequence divergence between specimen of nematodes is smaller than 0.5% in 450 nucleotide bases, these specimens should belong to the same 'molecular operational taxonomic unit' (MOTU). Many MOTUs defined in this study can be linked to the current taxonomic units based on morphologies. This study demonstrated the needs of a molecular identification database in facilitating the surveys in ecology, as well as the genetic diversity in natural environments.

Another study was conducted to demonstrate the feasibility of using 18S rRNA gene sequences as DNA barcode to discriminate terrestrial tardigrades (Blaxter *et al.*, 2004). Phylum Tardigrada is a neglected animal taxon due to its small body size. The body size of tardigrade ranging from less than 0.1 mm to about 1.5 mm, so it is

difficult to be identified based on its morphology. In this study, some MOTUs discovered can be correlated to different morphological taxa whereas several distinct MOTUs were referred to the same morphological tardigrade species. This indicated that cryptic species are present in tardigrades. Blaxter *et al.* (2004) concluded that 18S rRNA gene sequence can be used as DNA barcode for species identification of taxa such as tardigrade which is neglected and difficult to identify based on morphology only. DNA barcode, moreover, can facilitate the discovery and the presence of cryptic species in many groups of animal, and can reveal the biodiversity in natural environment.

It is suggested to use nuclear rRNA gene as DNA barcode for species identification, and to use mitochondrial rRNA gene for DNA barcoding. The performance of mitochondrial 16S rRNA gene sequences in DNA barcoding of amphibian was evaluated (Vences *et al.*, 2005). In this study, all the fresh and well-preserved samples of mantellid frog were successfully amplified by using single pair of 16S rRNA primers, there were only 50 to 70% of frog samples were successfully amplified by using three pairs of COI primers. The genetic divergence of 16S rRNA gene sequences among mantellid frog species ranged from 1 to 16.5%, while the intrapopulation divergences were ranging from 0 to 1%. Vences *et al.*

(2005) argued that this level of divergence among different frog species is suitable for assignment of frog larvae to certain species. In addition, Vences *et al.* (2005) compared the ability of 16S rRNA and COI gene sequences in discriminating eight selected vertebrate taxa. Results showed that 16S rRNA gene tree discriminated the eight taxa of vertebrates successfully, and this grouping was consistent with the current classification and phylogeny, while the COI gene tree can only recover two taxa of vertebrates, namely, cartilaginous fish and bird. Therefore, Vences *et al.* (2005) suggested that 16S rRNA gene sequence can be used as the universal maker in DNA barcoding not only for amphibians but also for other vertebrate groups.

2.3 Taxonomy of Nephropidae

2.3.1 Classification and phylogenetic relationship of Nephropidae

Clawed lobsters of family Nephropidae are a relatively small group of marine crustaceans dated from the Middle Jurassic. There are 52 known extant species and they are assigned to 13 genera (Tshudy and Babcock, 1997; Tshudy, 2003). Most of the extant nephropid lobsters are deep-water species. There are 48 nephropid lobsters recorded at 200 meters or deeper waters (Tshudy, 2003).

The main difference between clawed lobsters and spiny lobsters (Palinuridae) is

that the first pleopod of nephropid lobster is a large pectinate claw. The overall external morphology of nephropid lobsters is very similar to that of freshwater crayfish (families Astacidae and Parastacidae). The main difference between nephropid lobsters and freshwater crayfish is that the forth and the fifth thoracic segments are fused in nephropid lobsters, whereas the fifth thoracic segment is movable in crayfish species (Tshudy and Babcock, 1997).

The classification of Nephropidae today are mainly based on morphological characters (e.g. Manning, 1969; Jenkins, 1972; Bruce, 1988; Chan, 1997; Tshudy and Babcock, 1997; Tshudy *et al.*, unpublished). The description and discovery of Nephropidae species are also depended on external morphology (Chan and Yu, 1987; 1988; Chan *et al.*, 1991; Merino and Lindley, 2003). Limited studies have been conducted to elucidate the phylogenetic relationships of Nephropidae based on molecular data.

Based on morphological characteristics, it was that clawed lobsters are closely related to freshwater crayfish (families Astacidae and Parastacidae) (Hobbs, 1974). Recently, this hypothesis was supported by a molecular study (Crandall *et al.*, 2000). Phylogenetic tree inferred from small subunit ribosomal RNA (18S rRNA) gene

sequences supported the sister relationship between clawed lobsters and freshwater crayfish.

Tam and Kornfield (1998) elucidated the phylogenetic relationships of five genera in Nephropidae based on large subunit mitochondrial ribosomal RNA (16S rRNA) gene sequences. The results from this study are not consistent with the phylogeny inferred from morphological features. Based on morphological similarities, *Homarinus* was suggested to be closely related to *Homarus*, and *Metanephrops* is a sister genus to *Nephrops*. Molecular data suggested a different relationship between these genera. *Nephrops* and *Homarus* were found to be monophyletic while *Homarinus* were related to but outside the *Nephrops* and *Homarus* clade. It has been suggested that the morphological similarities between *Homarinus* and *Homarus*, as well as between *Metanephrops* and *Nephrops* was due to convergence or symplesiomorphy (Tam and Kornfield, 1998). Therefore, taxonomy and the phylogenetic relationships proposed based only on morphologies were doubted. Tam and Kornfield (1998) suggested to carry out further study that includes both molecular and morphological characters to infer the evolutionary history of clawed lobsters in Nephropidae.

In addition, molecular technique has been developed to aid in differentiating the two closely related species of Nephropidae (Hughes and Beaumont, 2004). Although there are characteristics to distinguish the American lobster *Homarus americanus* from the European lobster *H. gammarus*, these two lobsters cannot be distinguished solely based on their body tissues or claw morphology. Hughes and Beaumont (2004) reported that by using three sets of RAPD markers, these two species of nephropid lobsters can be discriminated. However, PCR products amplified by RAPD method sometimes were reported as unreliable and unrepeatable (Patwary *et al.*, 1994) and therefore, it is suggested to have further development of the methodology.

2.3.2 Classification and distribution of *Metanephrops*

Among 52 species in Nephropidae, 17 belong to genus *Metanephrops*. Although *Metanephrops* is the most specoise genus in Nephropidae, there are limited phylogenetic studies on *Metanephrops* and current proposed phylogeny in *Metanephrops* are solely based on morphology (Jenkins, 1972; Tshudy *et al.*, unpublished). Before 1972, the genus *Metanephrops* did not exist and clawed lobsters in '*Metanephrops*' were classified as *Nephrops*. Jenkins (1972) examined seven fossil specimens of clawed lobsters that were discovered at the South Island of New Zealand. He discovered that the fossil lobsters should belong to a new genus

which was closely related to the extant clawed lobsters occurred in the Indo-West Pacific region, the West Indies, and off the south-east coast of South America. As a result, he then accommodated these extent clawed lobsters into a new genus *Metanephrops* and *M. japonicus* was named as the type species of this genus. The lobster of genus *Nephrops* was distinguished from *Metanephrops* and confined to contain only an extant European species, *Nephrops norvegicus*.

Several authors have divided members of *Metanephrops* into four main groups (Table 2.1) based on their morphological features (e.g. De Man, 1916; Yaldwyn, 1954; Jenkins, 1972; Chan and Yu, 1987; Holthuis, 1991). Since the erection of *Metanephrops* in 1972, the number of extant species has increased from 13 to 17. During a revision of the Nephropidae in Taiwan (Chan and Yu, 1987), a new *Metanephrops* species, *M. formosanus,* was described. Another new species, *M. mozambicus*, was described during a study on a collection of Nephropidae from the Indian Ocean (Macpherson, 1990). In an enzyme polymorphism study of three *Metanephrops* species from Taiwan (Chu *et al.*, 1990), it has been suggested that *M. formosanus* and *M. japonicus var.* in Taiwan were closely related. The electrophoresis results provided evidence that the *M. formosanus* should belong to

Table 2.1 Four main groups of *Metanephrops*

Species group	Group member
arafurensis	*M. arafurensis*
	M. australiensis
	M. neptunus
binghami	*M. binghami*
	M. rubellus
japonicus	*M. andamanicus*
	M. armatus
	M. formosanus
	M. japonicus
	M. mozambicus
	M. sagamiensis
	M. velutinus
thomsoni	*M. boschmai*
	M. challengeri
	M. sibogae
	M. sinensis
	M. thomsoni

the '*japonicus*' group. Later on, during a study of some *japonicus* group members, Chan and Yu (1991) described the so called '*M. japonicus var.*' as a new species named *M. armatus*, and another new species *M. velutinus* was also described.

The extant *Metanephrops* are distributed between 50 and 1000 m, with most of the species living beneath 150 m (Jenkins, 1972; Chan, 1997; Tshudy, 2003). *Metanephrops* can be found in the outer edge of the continental shelf and the upper part of the continental slope (Jenkins, 1972; Chan, 1997).

Members of *Metanephrops* distribute widely in the Indo-West Pacific region (Jenkins, 1972; Chan, 1997). The *japonicus* group has the widest distribution range in the Indo-West Pacific region (Figure 2.1). The *thomsoni* group distributes in the western Pacific, and some members (e.g. *M. boschmai*, *M. challengeri* and *M. thomsoni*) can be found in western Australia. Yet the distribution of *arafurensis* group is restricted to the Philippine-Australian region, with one fossil species (*M. motunauensis*) in New Zealand. The *binghami* group is restricted to the western Atlantic, in where they can be found in the southeastern part of North America and the eastern part of South America.

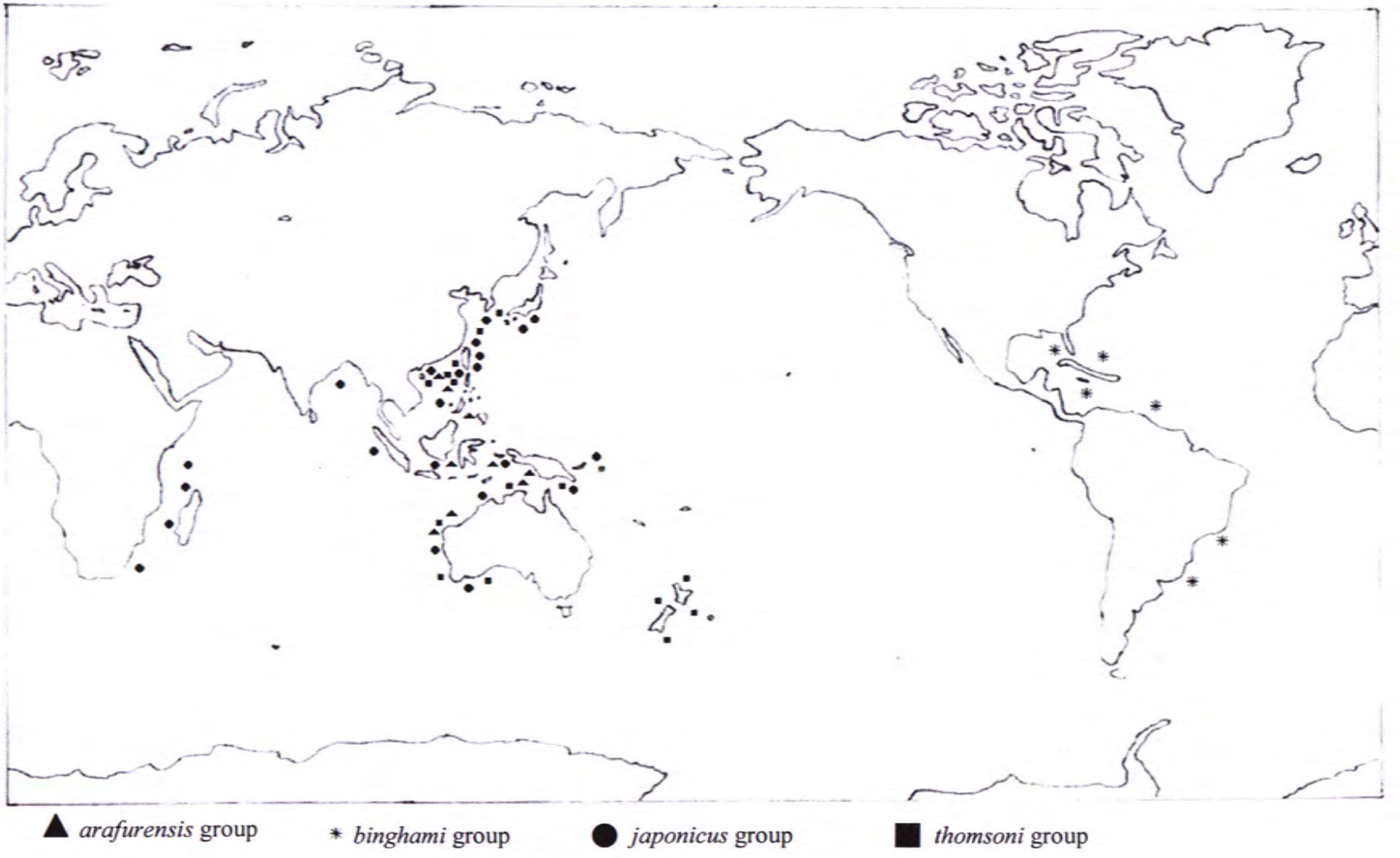

Figure 2.1 Geographical distribution of the four species groups of *Metanephrops*

2.3.3 Evolutionary history of *Metanephrops*

Due to the large number of extant species distribute in the Indo-West Pacific region, it has been hypothesized that the genus *Metanephrops* probably originated in this region (Jenkins, 1972). Since 11 out of 18 species of *Metanephrops* can be found in the Indo-Malay region, Chan (1997) suggested that the origin of *Metanephrops* was in the Indo-Malay region. The direct ancestor of *Metanephrops*, however, was not identified by Jenkins (1972) or Chan (1997). Based on the discovery of fossil *M. jenkinsi* at the Antarctic Peninsula and the morphological similarities between fossil records of *M. jenkinsi* and *Hoploparia stokesi*, Feldmann and Tshudy (1989) gave a different view and proposed that the genus *Metanephrops* evolved from the genus *Hoploparia* in the Antarctic region. Jenkins (1972) suggested two possible migration routes for the ancestor of *binghami* group from Indo-West Pacific region to Atlantic region, the ancestor either arrived Atlantic through the Tethys Sea or via southern Africa before or during the Lower Miocene. Jenkins (1972) noted that *binghami* group should arrive Atlantic through the Tethys Sea instead of migration via southern Africa. Chan (1997) also agreed with Jenkins' suggestion on the migration route of *binghami* species.

Phylogenetic relationships among members of *Metanephrops* have also been

inferred from their morphological features (Jenkins, 1972). The extant *japonicus* and *arafurensis* members include the most diverse species, suggest that these two groups are the oldest of the modern species groups. Moreover, Jenkins (1972) hypothesized that the species in *binghami* and *japonicus* groups shared a common ancestor. Jenkins (1972) also suggested that the *thomsoni* group is more closely related to *arafurensis* members, and proposed that species from *thomsoni* group might evolve from the ancestor of *M. australiensis* in the Indonesian region.

Chapter 3

Molecular Phylogeny of *Metanephrops*

3.1 Introduction

Clawed lobsters in *Metanephrops* have been divided into four morphological-based groups, namely, '*arafurensis*', '*binghami*', '*japonicus*' and '*thomsoni*' (Jenkins, 1972). Species from *Metanephrops* were under genus *Nephrops* before the erection of *Metanephrops,* it has been proposed that the genus was originated in the Indo-West Pacific region (Jenkins, 1972). Chan (1998) further proposed the place where *Metanephrops* originated at Indo-Malay region. However, the discoveries of some fossil records of *Metanephrops* at the Antarctic region made Feldmann and Tshudy (1989) proposed Antarctic is the region where *Metanephrops* was erected. Studies of the taxonomy and evolutionary history of *Metanephrops* were limited, and most of the studies were based mainly on morphological features (Jenkins, 1972; Chan, 1997; Tshudy *et al.*, unpublished). It has been noted that taxonomic classification based on morphological data cannot fully reflect the phylogenetic relationships of clawed lobster Nephropidae (Tam and Kornfield, 1998). Molecular and morphological data, therefore, have been suggested to complement each other for inferring the phylogeny of Nephropidae. The present study aims to elucidate the phylogenetic relationship of *Metanephrops* based on partial sequences

of two mitochondrial genes, the large subunit ribosomal RNA (16S rRNA) and cytochrome *c* oxidase subunit I (COI) genes, to evaluate the validity of the four morphological-based groups and to compare the previous proposed phylogenetic relationships among the four groups of *Metanephrops* (Figure 3.1).

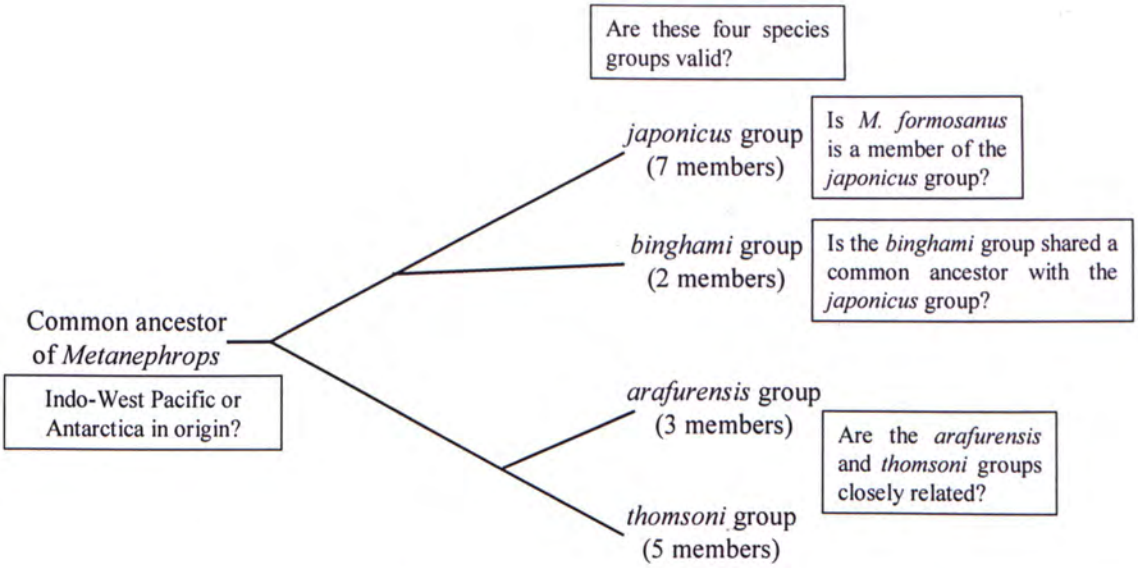

Figure 3.1 Phylogenetic relationship of *Metanephrops* inferred from morphology. Questions to be addressed in boxes in the present study using molecular data

3.2 Materials and Methods

3.2.1 Species studied and sample collection

Specimens of clawed lobsters were acquired from several collections from museums, including the National Taiwan Ocean University in Keelung, Taiwan, the Muséum national d'Histoire naturelle in Paris, France and the Nationaal Natuurhistorisch Museum in Leiden, the Netherlands. All of the samples were preserved in 70% ethanol when received. Some of the samples, however, were preserved previously in formalin during storage in museums. Sample collection localities and the present depository storage are listed in Table 3.1.

Table 3.1 Collecting localities and present depository of species used in this study

Species	Abbreviation	No. of individuals	Sampling locality (Present depository*)
M. andamanicus	M. and	2	Pratas, South China Sea (NTOU)
M. arafurensis	M. ara	1	Indonesia (MNHNP)
M. armatus	M. arm	1	Taiwan (NTOU)
M. australiensis	M. aus	2	Philippines (NTOU)
M. binghami	M. bin	1	NW coast of Panama, Mosqito Gulf (NNML)
M. challengeri	M. cha	3	One from New Zealand (MNHNP) and two acquired at Sydney fish market (NTOU)
M. formosanus	M. for	1	Taiwan (NTOU)
M. japonicus	M. jap	2	Sagami Bay, Japan (NTOU)
M. mozambicus	M. moz	1	Madagascar (MNHNP)
M. neptunus	M. nep	3	Philippines (MNHNP), Indonesia (MNHNP) and Pratas, South China Sea (NTOU)
M. rubellus	M. rub	1	Brazil (MNHNP)
M. sagamiensis	M. sag	3	Su-Aou, Taiwan (NTOU)
M. sibogae	M. sib	2	Indonesia (MNHNP) and Australia (NTOU)
M. sinensis	M. sin	1	Maribonoc Bay, Bohol, the Philippines (NTOU)
M. thomsoni	M. tho	4	One from the Philippines (NTOU) and three from Taiwan (NTOU)
M. velutinus	M. vel	1	Indonesia (MNHNP)
Acanthacaris tenuimana	A. ten	1	Solomon Island (MNHNP)
Homarus gammarus	H. gam	1	Paris supermarket (NTOU)

* MNHNP: Muséum national d'Histoire naturelle in Paris, France
NNML: Nationaal Natuurhistorisch Museum in Leiden, the Netherlands
NTOU: National Taiwan Ocean University in Keelung, Taiwan

3.2.2 DNA extraction

Total DNA of each individuals were extracted from one to two pleopods using the QIAamp® DNA Mini Kit (QIAGEN, Hilden, Germany). Procedures as recommended by the manufacturer were followed except the final step: 200 µl of double distilled water (ddH_2O) instead of 200 µl AE buffer was used to elute DNA from the spin column. After extraction, 5 µl of elutes were subjected to 1.5% agarose gel electrophoresis and ethidium bromide staining to access the quality of extracted DNA.

3.2.3 Amplification of mitochondrial genes

Partial segments from two mitochondrial genes, the large subunit ribosomal RNA (16S rRNA) and the cytochrome *c* oxidase subunit I (COI) genes, were amplified from the total DNA by polymerase chain reaction (Saiki *et al.*, 1988). Conserved primers were used to amplify these two segments of genes. The primer pair 16S ar (Simon *et al.*, 1994) and 16S 1472 (Crandall and Fitzpatrick, 1996) was used for amplifying the partial 16S rRNA gene (Table 3.2). The region amplified by this primer pair was near to the 3' end of the gene and the expected PCR product size was approximately 580 bp.

Table 3.2 Primer sequences used in the amplification and sequencing in this study

Primer name	Primer sequence 5'	3'
16S ar	CGCCTGTTTATCAAAAACAT	
16S 1472	AGATAGAAACCAACCTGG	
LCO 1490	GGTCAACAAATCATAAAGATATTGG	
HCO 2198	TAAACTTCAGGGTGACCAAAAAATCA	
COI f	CCTGCAGGAGGAGGAGAYCC	
COI a	AGTATAAGCGTCTGGGTAGTC	

Two primer pairs, LCO 1490 and HCO 2198 (Folmer *et al.*, 1994) and COI f and COI a (Palumbi and Benzie, 1991), were used for amplifying two regions of COI gene overlapped by 68 bp (Table 3.2). The primer pair LCO 1490 and HCO 2198 was used to amplify the 5' end of the COI gene and the expected PCR product size was approximately 700 bp. The 3' end of the COI gene region was amplified with the primer pair COI f and COI a and the expected PCR product size was approximately 660 bp.

The amplifications were set up in 50 µl reaction volume containing 2 µl of the DNA extract, 5 µl of 10x QIAGEN PCR buffer, 1.5-2.5 mM $MgCl_2$ (depending on gene), 0.2 µM of each primer, 0.2 mM of deoxynucleotide triphosphate mix (dNTPs), 1.5 units of *Taq* polymerase (5 units/µl) and ddH_2O added up to 50 µl reaction volume.

The PCR cycling profile for the amplification of 16S rRNA gene was performed with an initial denaturing step for 3 minutes at 94°C, followed by 33 cycles of 30 seconds at 94°C, annealing step for 30 seconds at 50°C and extension for 50 seconds at 72°C, and a final extension step at 72°C for 5 minutes.

The PCR cycling profile for the amplification of COI gene was performed with an initial denaturation step for 3 minutes at 94°C, followed by 33 cycles of 30 seconds at 94°C, annealing step for 50 seconds at 46°C and extension for 55 seconds at 72°C, and a final extension step at 72°C for 5 minutes.

3.2.4 Nucleotide sequencing

3.2.4.1 Asymmetric PCR

Prior to sequencing, PCR products were purified using QIAquick PCR purification kit (QIAGEN, Hilden, Germany). The purification procedures provided by the manufacturer were followed. Purified double-stranded PCR products were sequenced from both directions using each of the same primer pair (Table 3.2) for asymmetric PCR (cycle sequencing reactions) based on dideoxynucleotide chain termination reaction method (Sanger *et al.*, 1977). Cycle sequencing reaction mix contained 8 µl of ABI PRISM dRhodamine terminator reaction mix (Applied Biosystems, Foster City, California), 30 ng/µl of purified PCR product, 0.165 M of primer, and ddH_2O added up to total reaction volume 20 µl.

The cycling profile was as follows: 1 minute at 96°C, then 25 cycles of 30 seconds at 96°C, 15 seconds at 50°C, 4 minutes at 60°C and then kept at 4°C.

3.2.4.2 Purification of asymmetric PCR products

Ethanol-sodium acetate precipitation method was employed to remove the unincorporated primers and excess dye terminators. The sequencing reaction products were purified by adding 2 µl of 3 M sodium acetate (NaOAc, pH 4.6) and 50 µl of 95% ethanol. The mixture was put under ice for 10 minutes to precipitate the extension product. Pellet was obtained by centrifuging the mixture at 20000 rpm for 30 minutes. The pellet was rinsed with 500 µl of 70% ethanol and then centrifuged at 20000 rpm for 10 minutes. The products were vacuum dried and resuspended in 15 µl Hi-Di formamide (Applied Biosystems, Foster City, California). The purified products were denatured at 96°C for 4 minutes. Denaturation was quenched by storing the products under ice for 10 minutes. The denatured samples were then loaded onto an automatic sequencer (ABI PRISM® 3100 Genetic Analyzer, Applied Biosystems) for analyses.

3.2.5 Sequence alignment

Nucleotide sequences of each mitochondrial gene from each specimen were inspected with the aid of ABI Sequence Editor 1.0.3 (Applied Biosystems). Nucleotide sequences of each gene segment were checked by referring to the data from both strands of each individual. The two segments of COI sequenced were then

combined to a single gene sequence. All sequences of each gene were aligned by a built-in Clustal W 1.6 (Thompson *et al.*, 1994) program implemented in *MEGA* version 3.1 (Kumar *et al.*, 2004). In order to prevent the amplification of pseudogene sequences, all aligned and amplified COI gene sequences were confirmed by translating into amino acid sequences after alignment.

3.2.6 Phylogenetic analyses

A representative sequence from a single gene was chosen randomly for the subsequent phylogenetic analyses when sequence divergence between different specimens of the same species was less than 1%. Three data sets, sequences of 16S rRNA and COI genes and their combined sequences (including sequences of taxa which were available for both genes) were analyzed independently using PAUP* version 4.0 beta version 10 (Swofford, 2000). The partition homogeneity test (Farris *et al.*, 1995) for the combined data set was conducted using PAUP*.

Phylogenetic trees were constructed based on three methods, distance using BIO neighbor-joining (Gascuel, 1997), parsimony (Camin and Sokal, 1965) and maximum likelihood (Felsenstein, 1981) with PAUP*. Base composition and transition/transversion (ti/tv) ratios were calculated using PAUP*. The best-fitting

model of DNA substitution for the three data sets used in BIO neighbor-joining and maximum likelihood analyses were accessed by using the hierarchical likelihood ratio tests as implemented in Modeltest version 3.7 (Posada and Crandall, 1998).

Estimates of sequence divergence between species pairs based on uncorrected pairwise distance (p-distance) and the maximum likelihood method incorporating the best-fitting model of the data set were generated by PAUP*. For neighbor-joining analyses, pairwise deletion option was used for alignment gaps and missing information. 1000 bootstrap replicates were performed to access the confidence level at each branch (Felsenstein, 1985).

For parsimony and maximum likelihood analyses, heuristic searches were undertaken with 10 random addition sequence replicates and tree bisection-reconnection branch swapping. Gaps in sequences were treated as the fifth characters in parsimony analyses. All characters were weighted equally and only phylogenetically informative characters (Hillis *et al.*, 1996) were used in the analyses. 1000 and 250 bootstraps replicates were conducted to assess nodal support in parsimony and maximum likelihood analyses respectively.

In order to evaluate the degree of phylogenetic signal in all of the data set, the permutation tail probability (PTP) test (Faith and Cranston, 1991) as implemented in PAUP* was carried out to ensure that tree lengths were highly significant for all of the data sets ($P \leq 0.05$).

3.3 Results

3.3.1 PCR products of 16S rRNA and COI genes

The 16S rRNA gene segment was amplified from 16 species of *Metanephrops* and for each of the species, segments from one to four individuals were amplified (Table 3.3). Approximately 570 bp of 16S rRNA gene PCR products were sequenced from all specimens.

The amplifications of COI gene from five species of *Metanephrops*, namely *M. australiensis, M. binghami, M. challengeri, M. neptunus* and *M. rubellus*, were unsuccessful (Table 3.3). For the remaining 11 species, two segments of COI gene were amplified. For each of the species, sequences from one to four individuals were determined (Table 3.3). The size of PCR product amplified using primer pair LCO 1490/HCO 2198 was about 700 bp and the size of amplified PCR product using COI f/COI a was about 660 bp as shown in 1.5% agarose gel.

Table 3.3 Number of individuals sequenced for 16S rRNA and COI genes

Name of species	No. of individual sequenced	
	16S rRNA	COI
M. andamanicus	2	1
M. arafurensis	1	1
M. armatus	1	1
M. australiensis	2	-
M. binghami	1	-
M. challengeri	3	-
M. formosanus	1	1
M. japonicus	2	2
M. mozambicus	1	1
M. neptunus	2	-
M. rubellus	1	-
M. sagamiensis	3	2
M. sibogae	2	1
M. sinensis	1	1
M. thomsoni	4	4
M. velutinus	1	1
Acanthacaris tenuimana	1	1
Homarus gammarus	1	1

3.3.2 Nucleotide composition of 16S rRNA gene alignments

16S rRNA gene sequences with length of 488 to 491 bp were determined from specimens of *Metanephrops* (Table 3.4). The length of final aligned and truncated sequences was 497 bp (Appendix 1). There were 100 parsimony informative sites and 140 variable sites. The average base frequencies were 32.06% A, 11.26% C, 21.42% G and 35.26% T (Table 3.4). The A + T bases frequency is 67.32%, indicating that moderate AT bias in 16S rRNA gene of *Metanephrops*. This was consistent with pervious reports on base compositional bias in 16S rRNA sequences in crustaceans (Tam and Kornfield, 1998; Tong *et al.*, 2000; Ptacek *et al.*, 2001; Chu *et al.*, 2003; Ahyong and O'Meally, 2004; Harrison, 2004).

Table 3.4 Nucleotide frequencies of 16S rRNA gene sequences in *Metanephrops*

Species	A (%)	C (%)	G (%)	T (%)	(sequence length in bp)
M. andamanicus	31.63%	11.02%	22.25%	35.10%	490
M. arafurensis	31.90%	11.04%	21.27%	35.79%	489
M. armatus	31.43%	11.43%	22.86%	34.29%	490
M. australiensis	32.31%	11.04%	21.06%	35.58%	489
M. binghami	31.98%	12.63%	21.79%	33.61%	491
M. challengeri	33.40%	10.04%	18.85%	37.71%	488
M. formosanus	32.31%	11.45%	21.27%	34.97%	489
M. japonicus	31.43%	11.43%	22.65%	34.49%	490
M. mozambicus	31.43%	11.43%	22.25%	34.90%	490
M. neptunus (I)*	32.45%	10.20%	19.80%	37.55%	490
M. neptunus (P)**	32.25%	10.41%	20.00%	37.35%	490
M. rubellus	31.97%	12.71%	22.34%	32.99%	488
M. sagamiensis	31.63%	11.22%	22.25%	34.90%	490
M. sibogae	32.03%	11.91%	21.36%	34.70%	487
M. sinensis	31.97%	11.48%	21.11%	35.45%	488
M. thomsoni (P)#	32.45%	11.02%	21.22%	35.31%	490
M. thomsoni (T)##	32.65%	11.22%	21.02%	35.10%	490
M. velutinus	31.84%	11.02%	22.25%	34.90%	490
Average	32.06%	11.26%	21.42%	35.26%	489.39

* *M. neptunus* (I): *Metanephrops neptunus* collected from Indonesia

** *M. neptunus* (P): *Metanephrops neptunus* collected from Pratas

\# *M. thomsoni* (P): *Metanephrops thomsoni* collected from the Philippines

\#\# *M. thomsoni* (T): *Metanephrops thomsoni* collected from Taiwan

3.3.3 Nucleotide composition of COI gene alignments

The two overlapping segments of COI genes were combined together for phylogenetic analyses. The length of aligned combined and truncated sequences for *Metanephrops* species was 1221 bp in length (Table 3.5). Of these, 300 sites were parsimony informative and there were 431 variable sites. The average base frequencies are 24.96% A, 24.07% C, 18.01% G and 32.96% T (Table 3.5). The moderate AT bias (A+T% = 57.92%) of COI gene sequences in *Metanephrops* was consistent with pervious reported in base compositional bias in COI gene sequences in crustaceans (Chu *et al.*, 1999; Tong *et al.*, 2000; Ptacek *et al.*, 2001; Chu *et al.*, 2003; Harrison, 2004).

Table 3.5 Nucleotide frequencies of COI gene sequences in *Metanephrops* (1221 bp)

Species	A (%)	C (%)	G (%)	T (%)
M. andamanicus	24.71%	24.30%	18.47%	32.51%
M. arafurensis	25.80%	22.85%	17.36%	34.00%
M. armatus	25.53%	24.24%	17.61%	32.60%
M. formosanus	24.73%	24.40%	18.43%	32.43%
M. japonicus	25.23%	25.14%	17.72%	31.86%
M. mozambicus	24.82%	24.73%	18.76%	31.70%
M. sagamiensis	25.06%	23.75%	18.63%	32.51%
M. sibogae	24.82%	24.08%	18.35%	32.76%
M. sinensis	24.49%	24.32%	18.51%	32.68%
M. thomsoni (P)*	24.98%	23.67%	17.53%	33.83%
M. thomsoni (T)**	24.90%	23.59%	17.72%	33.74%
M. velutinus	24.82%	23.83%	18.76%	32.60%
Average	24.96%	24.07%	18.01%	32.96%

* *M. thomsoni* (P): *Metanephrops thomsoni* collected from the Philippines

** *M. thomsoni* (T): *Metanephrops thomsoni* collected from Taiwan

3.3.4 Intraspecific and interspecific genetic variation

For the 16S rRNA gene segments, sequence divergence between conspecific individuals ranged from 0 to 0.2%, except in *M. neptunus* and *M. thomsoni*. Sequence divergence between specimens of *M. neptunus* collected from Indonesia and Pratas was 1.2% (Table 3.6). Sequence divergence between the four specimens of *M. thomsoni* collected from Taiwan were 0.2%, whereas sequence divergence between *M. thomsoni* collected from the Philippines and Taiwan ranged from 1 to 1.2% (Table 3.6). Having sequence divergences larger than 1%, the sequences of *M. neptunus* and *M. thomsoni* from different sample locations, therefore, were included in the phylogenetic analyses. For species with sequence divergence smaller than 1% among conspecific individuals, only one representative sequence of the species was included in the phylogenetic analyses.

Within the ingroup taxa, sequence divergence of 16S rRNA gene ranged from 0.2% between *M. andamanicus* and *M. velutinus* to 15.6% between *M. neptunus* collected form Pratas and *M. rubellus*, with a mean value of 7.3%. The sequence divergence ranged from 11.9 to 20.1% between ingroup taxa and the two outgroups (Table 3.6). For sequence divergence within members of the four main groups (Table 3.8), the highest was *arafurensis* (10.0%) and the lowest was *japonicus* group (0.2%).

For sequence divergence among the four main groups of *Metanephrops* (Table 3.8), the highest was between *arafurensis* and *binghami* groups (15.6%) while the lowest was between *japonicus* and *thomsoni* groups (2.7%).

Except for *M. thomsoni*, the conspecific sequence divergence of COI gene ranged from 0.2 to 0.3%. Sequence divergence between specimens of *M. thomsoni* collected from Taiwan ranged from 0 to 0.2% while the sequence divergence between *M. thomsoni* colleted from the Philippines and Taiwan was 3.6% (Table 3.7). Having sequence divergences larger than 1%, the sequences of *M. thomsoni* from the two sample locations were included in the phylogenetic analyses.

Within the ingroup taxa, the sequence divergences of COI gene ranged from 2.2% between *M. sagamiensis* and *M. velutinus* to 18.5% between *M. sagamiensis* and *M. sibogae* with a mean value of 10.9%, whilst those between ingroup taxa and the two outgroups were from 20.2 to 26.6% (Table 3.7). No COI gene sequence could be amplified from species in *binghami* group. For the COI sequence divergence among the three main groups of *Metanephrops* (Table 3.8), the highest and lowest sequence divergence were also between *japonicus* and *thomsoni* groups, 18.5 and 8.6%, respectively.

Table 3.6 Uncorrected p-distance (above diagonal) and Hasegawa, Kishino, Yano 85 model (Hasegawa *et al.*, 1985; below diagonal) estimates of sequence divergence based on 497 bp of 16S rRNA gene sequences among species of *Metanephrops* and the two outgroups. See Table 3.1 for species abbreviation.

	M. and	*M. ara*	*M. arm*	*M. aus*	*M. bin*	*M. cha*	*M. for*	*M. jap*	*M. moz*	*M. nep* (I)	*M. nep* (P)	*M. rub*	*M. sag*	*M. sib*	*M. sin*	*M. tho* (P)	*M. tho* (T)	*M. vel*	*A. ten*	*H. gam*
M. and	-	0.0416	0.0291	0.0416	0.1102	0.0728	0.0208	0.0291	0.0062	0.0748	0.0811	0.1164	0.0021	0.0561	0.0395	0.0333	0.0333	0.0021	0.1310	0.1268
M. ara	0.0468	-	0.0499	0.0083	0.1164	0.0665	0.0395	0.0499	0.0457	0.0811	0.0873	0.1310	0.0416	0.0437	0.0624	0.0457	0.0457	0.0437	0.1414	0.1331
M. arm	0.0315	0.0555	-	0.0499	0.1206	0.0832	0.0249	0.0083	0.0353	0.0832	0.0894	0.1206	0.0270	0.0603	0.0499	0.0437	0.0437	0.0312	0.1331	0.1310
M. aus	0.0469	0.0082	0.0555	-	0.1123	0.0665	0.0395	0.0499	0.0457	0.0811	0.0832	0.1331	0.0416	0.0395	0.0624	0.0499	0.0499	0.0437	0.1372	0.1310
M. bin	0.1226	0.1301	0.1351	0.1251	-	0.1185	0.1081	0.1185	0.1060	0.1143	0.1185	0.0832	0.1102	0.1143	0.1102	0.1164	0.1060	0.1123	0.1414	0.1455
M. cha	0.0812	0.0764	0.0927	0.0765	0.1355	-	0.0686	0.0811	0.0769	0.0416	0.0499	0.1268	0.0748	0.0790	0.0769	0.0728	0.0728	0.0748	0.1060	0.1102
M. for	0.0230	0.0424	0.0251	0.0424	0.1178	0.0765	-	0.0249	0.0270	0.0665	0.0728	0.1206	0.0187	0.0541	0.0333	0.0270	0.0270	0.0229	0.1206	0.1247
M. jap	0.0315	0.0555	0.0082	0.0556	0.1327	0.0905	0.0251	-	0.0353	0.0811	0.0873	0.1227	0.0270	0.0603	0.0499	0.0437	0.0437	0.0312	0.1331	0.1310
M. moz	0.0082	0.0535	0.0402	0.0535	0.1198	0.0884	0.0316	0.0402	-	0.0790	0.0852	0.1164	0.0083	0.0561	0.0457	0.0395	0.0395	0.0083	0.1310	0.1268
M. nep (I)	0.0838	0.0931	0.0928	0.0933	0.1300	0.0427	0.0742	0.0906	0.0909	-	0.0125	0.1247	0.0769	0.0748	0.0748	0.0707	0.0665	0.0769	0.1060	0.1102
M. nep (P)	0.0908	0.1001	0.0999	0.0953	0.1352	0.0516	0.0810	0.0976	0.0979	0.0124	-	0.1331	0.0832	0.0832	0.0811	0.0769	0.0728	0.0832	0.1060	0.1143
M. rub	0.1318	0.1470	0.1363	0.1500	0.0913	0.1480	0.1347	0.1393	0.1341	0.1451	0.1555	-	0.1185	0.1331	0.1227	0.1268	0.1185	0.1185	0.1518	0.1726
M. sag	0.0021	0.0468	0.0293	0.0469	0.1226	0.0837	0.0209	0.0294	0.0103	0.0862	0.0932	0.1344	-	0.0561	0.0416	0.0333	0.0333	0.0042	0.1310	0.1268
M. sib	0.0609	0.0453	0.0652	0.0409	0.1276	0.0893	0.0564	0.0654	0.0631	0.0840	0.0934	0.1498	0.0609	-	0.0603	0.0603	0.0644	0.0582	0.1414	0.1393
M. sin	0.0425	0.0672	0.0513	0.0673	0.1202	0.0858	0.0337	0.0514	0.0491	0.0834	0.0904	0.1365	0.0447	0.0630	-	0.0457	0.0457	0.0416	0.1185	0.1268
M. tho (P)	0.0381	0.0492	0.0469	0.0539	0.1311	0.0836	0.0273	0.0470	0.0470	0.0813	0.0882	0.1427	0.0381	0.0636	0.0470	-	0.0125	0.0353	0.1164	0.1268
M. tho (T)	0.0381	0.0492	0.0469	0.0538	0.1179	0.0835	0.0273	0.0470	0.0470	0.0764	0.0833	0.1318	0.0381	0.0684	0.0470	0.0124	-	0.0353	0.1143	0.1164
M. vel	0.0020	0.0490	0.0336	0.0490	0.1250	0.0835	0.0251	0.0336	0.0103	0.0861	0.0930	0.1342	0.0041	0.0632	0.0446	0.0403	0.0403	-	0.1331	0.1289
A. ten	0.1539	0.1638	0.1560	0.1585	0.1645	0.1198	0.1375	0.1564	0.1513	0.1198	0.1194	0.1756	0.1538	0.1624	0.1325	0.1323	0.1296	0.1564	-	0.1185
H. gam	0.1450	0.1498	0.1498	0.1473	0.1652	0.1223	0.1396	0.1501	0.1473	0.1219	0.1267	0.2010	0.1449	0.1563	0.1423	0.1423	0.1292	0.1474	0.1317	-

Table 3.7 Uncorrected p-distance (above diagonal) and Tamura-Nei model (Tamura and Nei, 1993; below diagonal) estimates of sequence divergences based on 1221 bp of COI gene sequences among species of *Metanephrops* and the two outgroups. See Table 3.1 for species abbreviation.

	M. and	*M. ara*	*M. arm*	*M. for*	*M. jap*	*M. moz*	*M. sag*	*M. sib*	*M. sin*	*M. tho (P)*	*M. tho (T)*	*M. vel*	*A. ten*	*H. gam*
M. and	-	0.1437	0.0685	0.0986	0.0685	0.0660	0.0343	0.1512	0.0802	0.0927	0.0927	0.0351	0.1988	0.2178
M. ara	0.1691	-	0.1270	0.1445	0.1337	0.1395	0.1437	0.1094	0.1320	0.1295	0.1337	0.1437	0.2080	0.1646
M. arm	0.0711	0.1510	-	0.0944	0.0301	0.0785	0.0777	0.1395	0.0902	0.0944	0.0919	0.0794	0.2013	0.1796
M. for	0.1066	0.1728	0.1017	-	0.1019	0.0927	0.0927	0.1504	0.0860	0.0927	0.0961	0.0902	0.2030	0.1830
M. jap	0.0709	0.1587	0.0325	0.1126	-	0.0769	0.0802	0.1429	0.0944	0.0961	0.0977	0.0835	0.1988	0.1888
M. moz	0.0695	0.1654	0.0857	0.1034	0.0846	-	0.0610	0.1362	0.0886	0.0977	0.1003	0.0585	0.1997	0.1880
M. sag	0.0361	0.1701	0.0847	0.1035	0.0863	0.0639	-	0.1546	0.0894	0.0911	0.0936	0.0217	0.1980	0.1805
M. sib	0.1768	0.1278	0.1655	0.1760	0.1703	0.1608	0.1849	-	0.1437	0.1353	0.1370	0.1520	0.2164	0.1746
M. sin	0.0863	0.1574	0.1009	0.0959	0.1026	0.0981	0.0973	0.1706	-	0.0744	0.0752	0.0869	0.2139	0.1788
M. tho (P)	0.1002	0.1527	0.1039	0.1013	0.1065	0.1092	0.1018	0.1569	0.0814	-	0.0334	0.0911	0.2038	0.1738
M. tho (T)	0.1022	0.1573	0.1030	0.1061	0.1084	0.1140	0.1046	0.1621	0.0823	0.0361	-	0.0944	0.2114	0.1788
M. vel	0.0370	0.1699	0.0865	0.1005	0.0899	0.0612	0.0216	0.1815	0.0943	0.1016	0.1054	-	0.1997	0.1830
A. ten	0.2374	0.2549	0.2438	0.2465	0.2390	0.2409	0.2381	0.2660	0.2603	0.2474	0.2576	0.2404	-	0.2147
H. gam	0.2284	0.2018	0.2223	0.2279	0.2363	0.2323	0.2238	0.2163	0.2225	0.2151	0.2230	0.2273	0.2664	-

Table 3.8 Sequences divergences among and between the four main groups of *Metanephrops*. Values for 16S rRNA and COI genes are shown before and after the dashes or above diagonal and below diagonal, respectively

Group	*arafurensis*	*binghami*	*japonicus*	*thomsoni*
arafurensis	0.8 – 10.0%/ N/A	13.0 – 15.6%	4.2 – 10.0%	4.1 – 9.3%
binghami	N/A	9.1%/N/A	11.8 – 13.9%	11.8 – 15.0%
japonicus	15.1 – 17.3%	N/A	0.2 – 4.0%/ 2.2 – 10.7%	2.7 – 9.3%
thomsoni	12.8 – 15.7%	N/A	8.6 – 18.5%	4.7 – 8.9%/ 8.1 – 17.1%

3.3.5 Phylogenetic analysis based on 16S rRNA gene sequences

Modeltest 3.7 suggested that the best-fitting model of substitution for the 16S rRNA data set was Hasegawa, Kishino, Yano 85 model (Hasegawa *et al.*, 1985) with a gamma distribution (HKY + G). Transition/transversion (ti/tv) ratio within the 16S rRNA gene sequences was 4.0262 (Table 3.9). The PTP test confirmed that tree lengths of the 16S rRNA data set were highly significant (P = 0.01).

Neighbor-joining (NJ) and maximum likelihood (ML) analyses determined from 16S rRNA gene sequences constructed identical tree topology (Figures 3.2 and 3.3), while maximum parsimony (MP) analysis generated a similar tree topology (Figure 3.4) to those of the NJ and MP trees. The monophyly of genus *Metanephrops* as compared with the two outgroups, *A. tenuimana* and *H. gammarus*, was supported by 98, 78 and 78% bootstrap proportions (BP) in NJ, ML and MP analyses, respectively.

Among *Metanephrops*, two major clades were derived. The clade containing *M. challengeri* (*thomsoni* group) and the two specimens of *M. neptunus* (*arafurensis* group) was supported by BP of 90, 73 and 73% in NJ, ML and MP trees, respectively. Supported by high bootstrap values (BP ≥ 91% in all trees), two specimens of *M.*

Table 3.9 Maximum likelihood parameters of best-fitting DNA substitution models used in the phylogenetic analyses of *Metanephrops*

Gene	16S rRNA	COI	16S rRNA + COI
Best-fitting model	HKY+G*	TrN+I+G**	TrN+I+G**
Base frequencies			
A	0.3457	0.2815	0.3008
C	0.0906	0.2381	0.1999
G	0.1877	0.1426	0.1577
T	0.3760	0.3377	0.3416
Substitution rate			
A↔C	-	1.0000	1.0000
A↔G	-	14.5292	11.5715
A↔T	-	1.0000	1.0000
C↔G	-	1.0000	1.0000
C↔T	-	10.4219	11.0573
G↔T	-	1.0000	1.0000
Proportion of invariable sites	-	0.5227	0.5272
Gamma distribution shape parameter	0.1542	0.6000	0.5460
Transition/transversion ratio	4.0262	-	-

* Hasegawa, Kishino, Yano 85 model (Hasegawa *et al.*, 1985) with a gamma distribution

** Tamura-Nei model (Tamura and Nei, 1993) with a proportion of invariable sites and with a gamma distribution

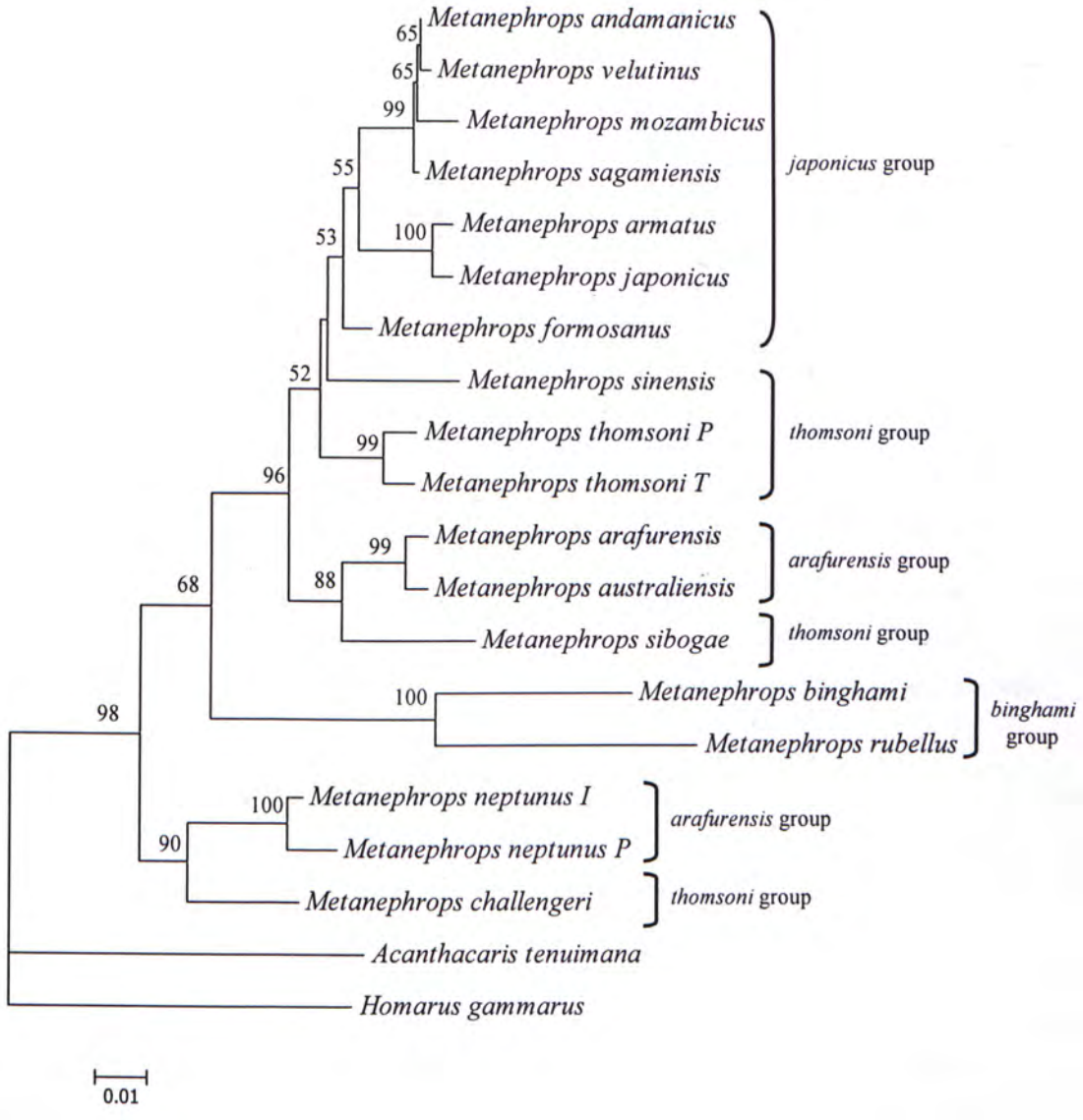

Figure 3.2 Neighbor-joining tree derived from 16S rRNA gene sequences. Numbers above branches are bootstrap values (1000 replicates). Bootstrap values below 50% are not shown.

Metanephrops neptunus I: *M. neptunus* collected from Indonesia
Metanephrops neptunus P: *M. neptunus* collected from Pratas
Metanephrops thomsoni P: *M. thomsoni* collected from the Philippines
Metanephrops thomsoni T: *M. thomsoni* collected from Taiwan

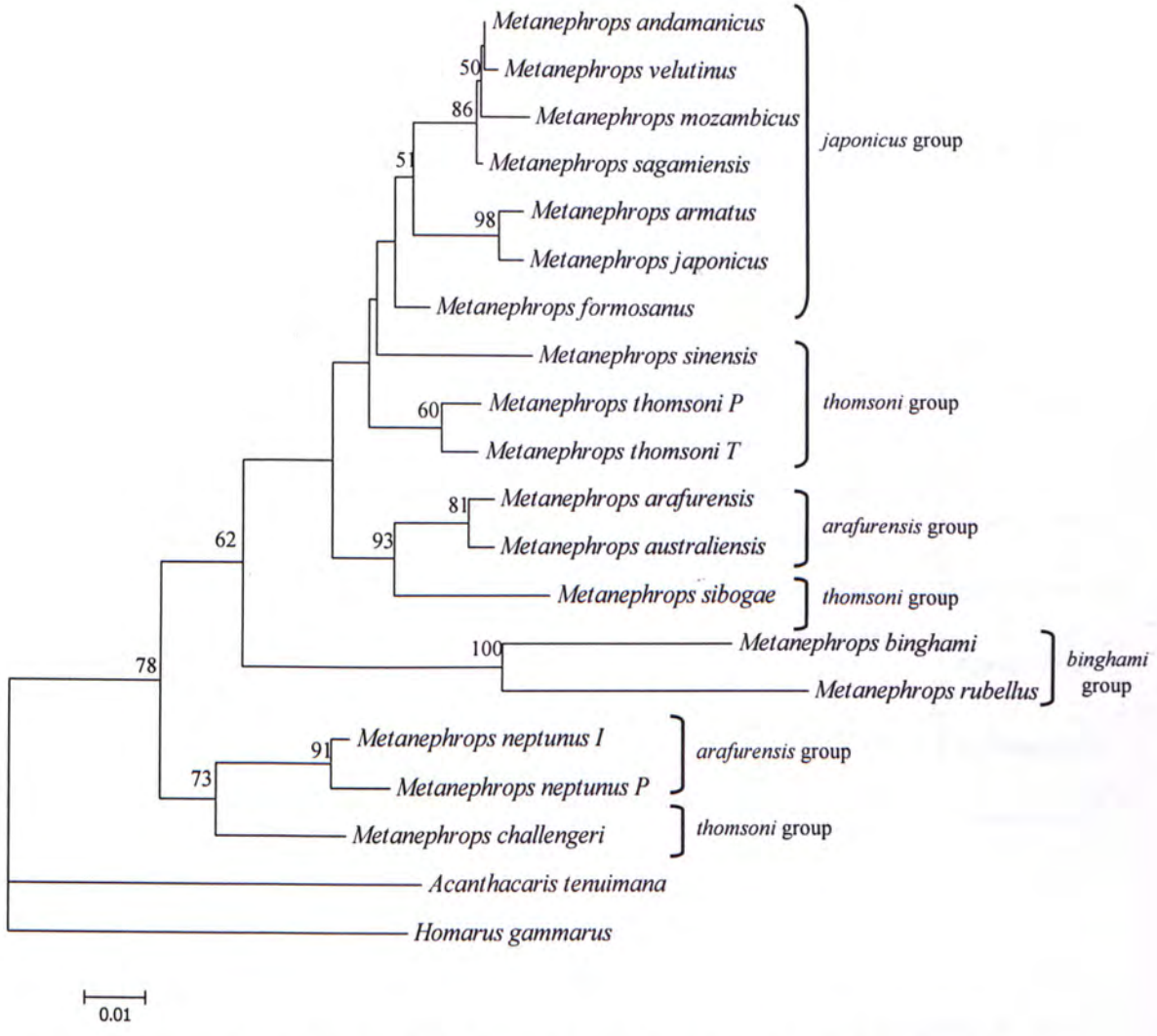

Figure 3.3 Maximum likelihood tree derived from 16S rRNA gene sequences. Numbers above branches are bootstrap values (500 replicates). Bootstrap values below 50% are not shown.

Metanephrops neptunus I: *M. neptunus* collected from Indonesia
Metanephrops neptunus P: *M. neptunus* collected from Pratas
Metanephrops thomsoni P: *M. thomsoni* collected from the Philippines
Metanephrops thomsoni T: *M. thomsoni* collected from Taiwan

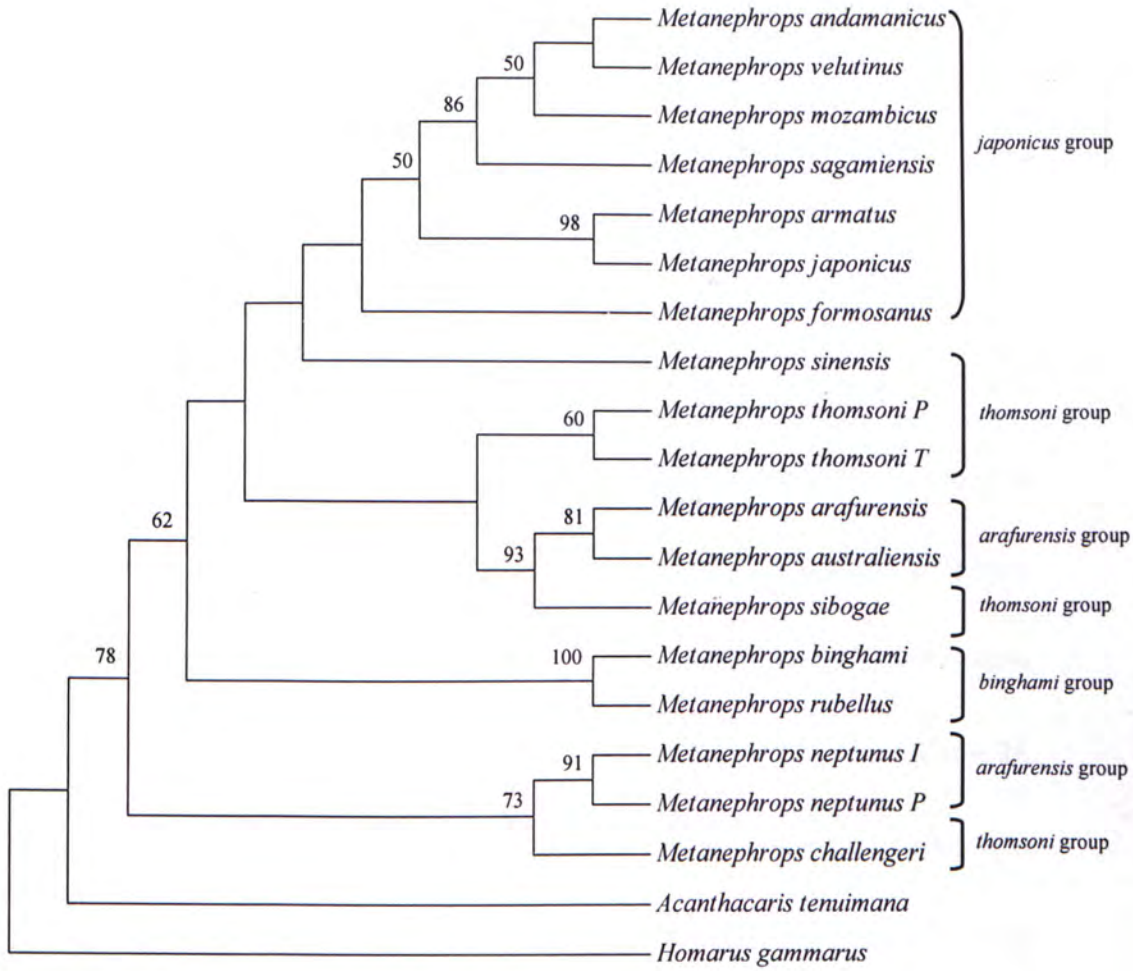

Figure 3.4 Maximum parsimony tree derived from 16S rRNA gene sequences. Numbers above branches are bootstrap values (1000 replicates). Bootstrap values below 50% are not shown.

Metanephrops neptunus I: *M. neptunus* collected from Indonesia
Metanephrops neptunus P: *M. neptunus* collected from Pratas
Metanephrops thomsoni P: *M. thomsoni* collected from the Philippines
Metanephrops thomsoni T: *M. thomsoni* collected from Taiwan

neptunus collected from different localities were more closely related to each other than to *M. challengeri*. Another clade containing the remaining *Metanephrops* species (i.e. all species from *binghami* and *japonicus* groups, as well as other members from the *arafurensis* and *thomsoni* groups including *M. arafurensis*, *M. australiensis*, *M. sibogae*, *M. sinensis* and *M. thomsoni*) was supported by 68, 62 and 62% in NJ, ML and MP analyses, respectively. Within this clade, the monophyly of the two species from *binghami* group (*M. binghami* and *M. rubellus*) was supported by high bootstrap values (BP = 100% in all trees). In NJ tree (Figure 3.2), BP of 96% supported all species from the *japonicus* group, *M. arafurensis*, *M. australiensis*, *M. sibogae* and *M. thomsoni* were clustered together. However, this relationship was only supported by low BP values of < 50% in ML and MP analyses (Figures 3.3 and 3.4). The two members of *arafurensis* group (*M. arafurensis* and *M. australiensis*) were closely related to each other (supported by high bootstrap values, BP ≥ 81% in all trees) and they formed a clade with *M. sibogae* (*thomsoni* group) supported by high confidence levels (BP ≥ 88% in all trees).

Similar to *M. neptunus*, the two specimens of *M. thomsoni* collected from different localities were grouped together with high BP support (99, 60 and 60% in NJ, ML and MP trees, respectively). In NJ tree (Figure 3.2), the two specimens of *M.*

thomsoni were then related to the clade containing *M. sinensis* and all species from *japonicus* group (BP = 52%). The ML tree (Figure 3.3) also showed the same pattern as the NJ tree (Figure 3.2), but the BP was low (< 50%). The MP tree (Figure 3.4), on the contrary, showed that the two specimens of *M. thomsoni* were closely related to *M. arafurensis*, *M. australiensis* and *M. sibogae* instead, but the bootstrap support was low (BP < 50%).

All analyses indicated that *M. sinensis* (*thomsoni* group) was closely related to all species from *japonicus* group, but only with low BP of < 50% supported this relationship. In all trees based on 16S rRNA gene sequences (Figures 3.2 to 3.4), *M. andamanicus* and *M. velutinus* were closely related to each other and they were more related to *M. mozambicus* than to *M. sagamiensis*, but the BP support these relationships in most cases were weak (BP of 65, 55% or below). In addition, *M. armatus* and *M. japonicus* were closely related to each other with high BP support in all analyses (BP ≥ 98 %). Moreover, the affinity of *M. formosanus* to other species in *japonicus* group was supported but only with weak BP (53% in NJ tree and < 50% in MP and ML trees).

3.3.6 Phylogenetic analysis based on COI gene sequences

The best-fitting model calculated by Modeltest 3.7 for COI gene data set was Tamura-Nei model (Tamura and Nei, 1993) with a proportion of invariable sites and with a gamma distribution (TrN + I + G; Table 3.9). The PTP test confirmed that tree lengths of the COI gene data set were highly significant (P = 0.01).

The phylogenetic relationships suggested by COI gene sequences analyses were similar to those based on 16S rRNA gene sequences. The topology determined from COI gene sequences based on NJ and ML analyses was identical (Figures 3.5 and 3.6), whilst the topology inferred by MP analysis was similar (Figure 3.7) to those of the NJ and ML analyses. The monophyletic relationship of genus *Metanephrops* from the two outgroups, *A. tenuimana* and *H. gammarus*, was supported by BP of 76, 59 and 96% in NJ, ML and MP analyses, respectively.

Among the ingroups taxa of *Metanephrops*, all taxa included in the COI analyses were divided into two major clades. The first clade consisted of *M. arafurensis* (*arafurensis* group) and *M. sibogae* (*thomsoni* group), supported by BP values of ≥ 80% in all trees. The close relationship between these two species was also supported in the phylogenetic trees based on 16S rRNA gene analyses (Figures

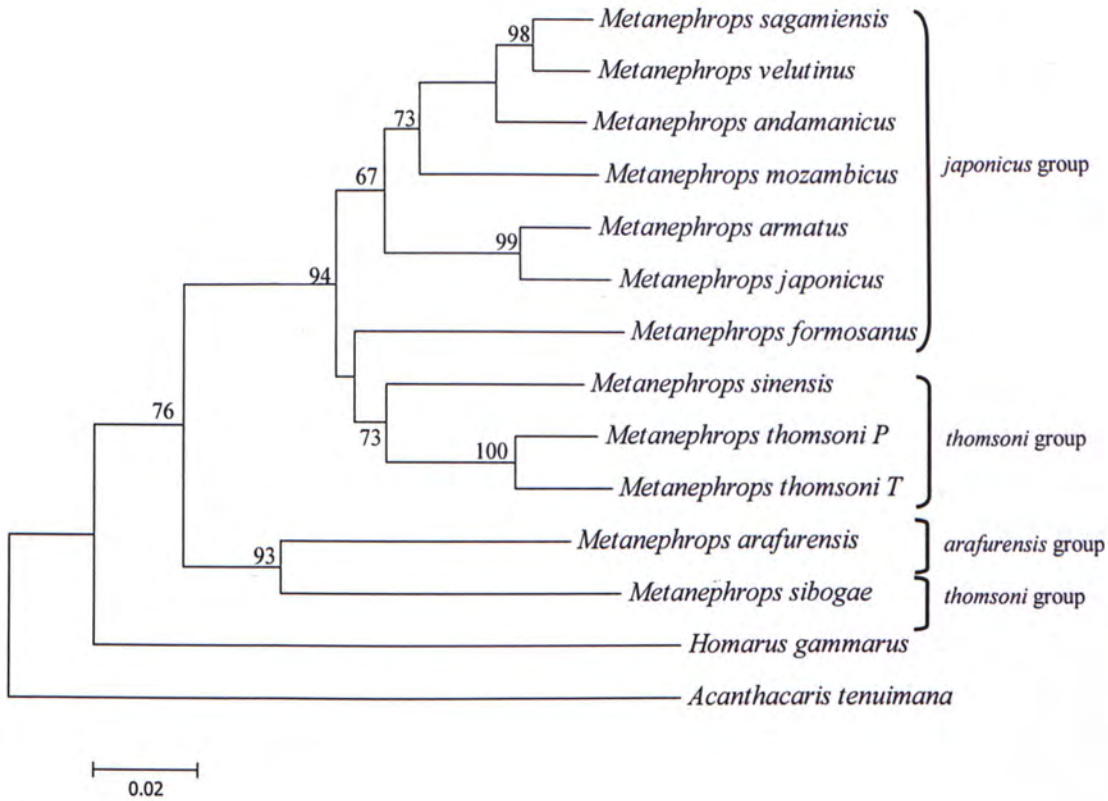

Figure 3.5 Neighbor-joining tree derived from COI gene sequences. Numbers above branches are bootstrap values (1000 replicates). Bootstrap values below 50% are not shown.

Metanephrops thomsoni P: *M. thomsoni* collected from the Philippines

Metanephrops thomsoni T: *M. thomsoni* collected from Taiwan

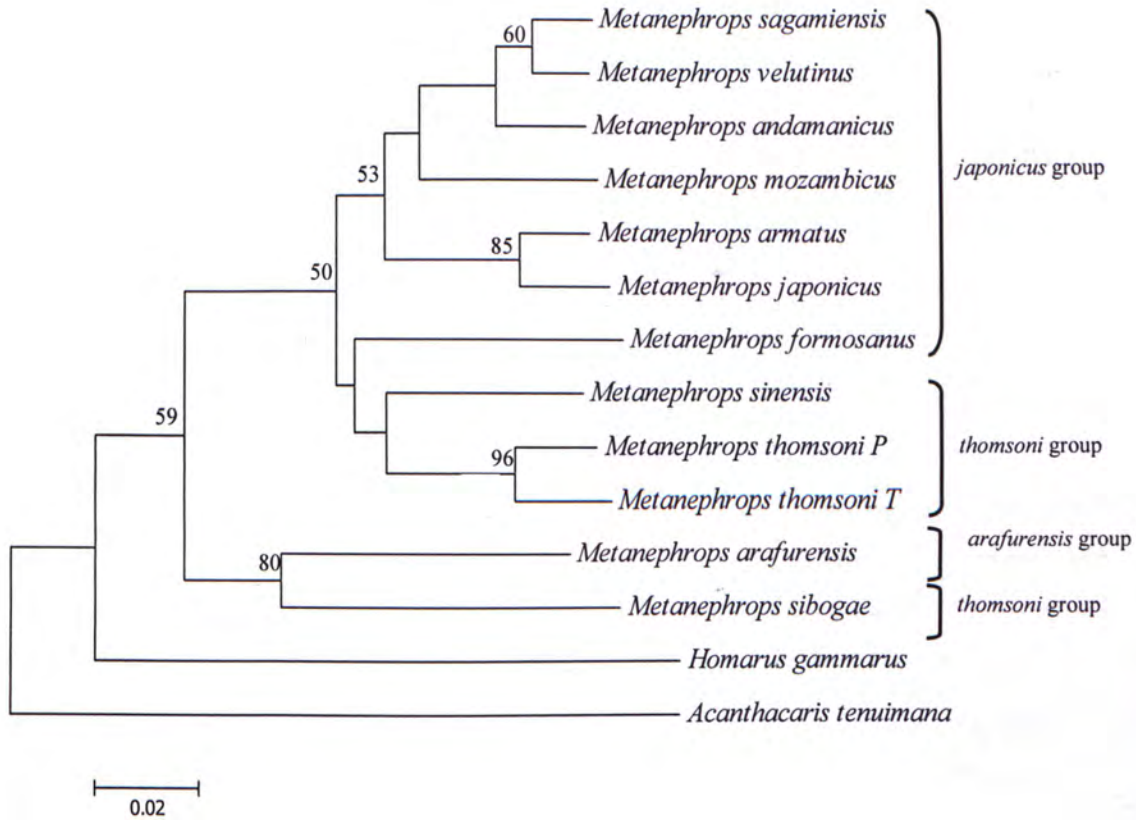

Figure 3.6 Maximum likelihood tree derived from COI gene sequences. Numbers above branches are bootstrap values (500 replicates). Bootstrap values below 50% are not shown.

Metanephrops thomsoni P: *M. thomsoni* collected from the Philippines
Metanephrops thomsoni T: *M. thomsoni* collected from Taiwan

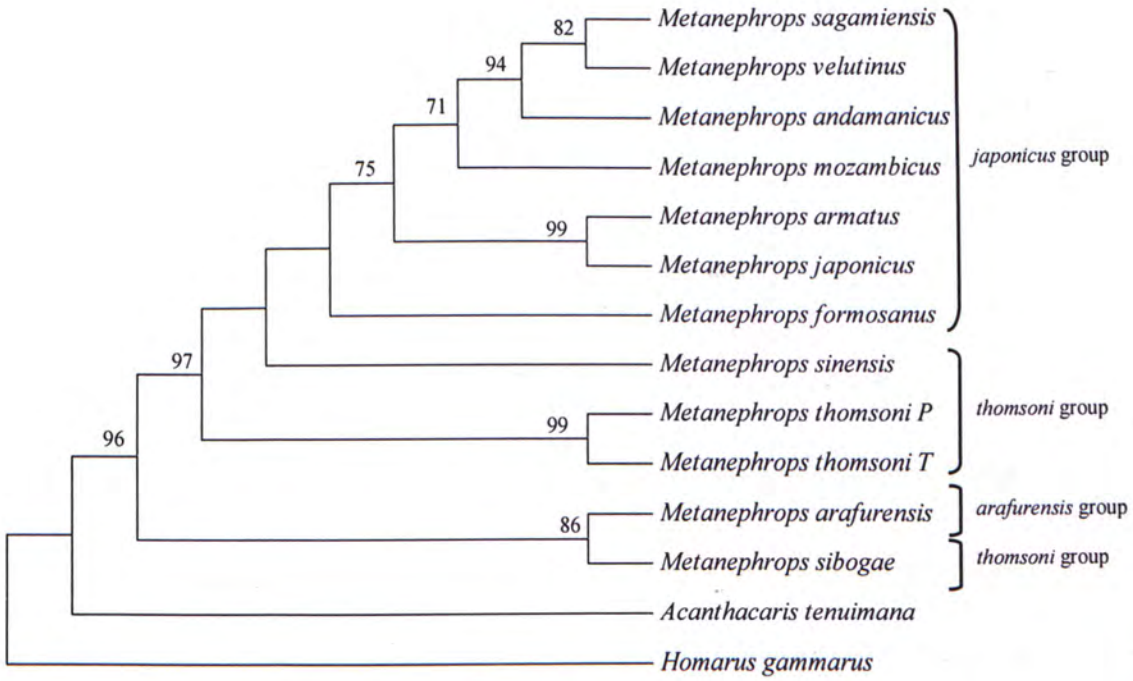

Figure 3.7 Parsimony tree derived from COI gene sequences. Numbers above branches are bootstrap values (1000 replicates). Bootstrap values below 50% are not shown.

Metanephrops thomsoni P: *M. thomsoni* collected from the Philippines
Metanephrops thomsoni T: *M. thomsoni* collected from Taiwan

3.2 to 3.4). The second clade contains the remaining species in the COI analyses (i.e. all species from *japonicus* group, *M. sinensis* and *M. thomsoni*) supported by BP values of 94, 50 and 97% in NJ, ML and MP trees, respectively.

The three analyses based on COI gene sequences supported the close relationship between two *M. thomsoni* (*thomsoni* group) specimens collected from different localities (supported by BP ≥ 96% in all trees). In the NJ tree (Figure 3.5), *M. thomsoni* was more related to *M. sinensis* (*thomsoni* group) with BP support of 73% than to *M. formosanus* with low BP support (BP < 50%). The ML tree (Figure 3.6) showed the same relationship but only with low bootstrap support (< 50%). The MP tree (Figure 3.7), in contrast, suggested that *M. sinensis* was more closely related to species in the *japonicus* group, but this was supported by BP < 50%.

Similar to the inference based on 16S rRNA gene sequences, COI data set also suggested that all the species from *japonicus* group, other than *M. formosanus*, were closely related, supported by BP values of 67, 53 and 75% in NJ, ML and MP trees, respectively. The affinity of *M. formosanus* to other species in *japonicus* group was supported but only with weak BP (< 50% in MP tree). The remaining species in *japonicus* group were divided into two clades. The first clade consisted of *M.*

armatus and *M. japonicus*, supported by high confidence levels (BP ≥ 85%) in all analyses. The second clade (supported by BP of 73, < 50 and 71% in NJ, ML and MP trees, respectively) of *japonicus* group contains *M. andamanicus*, *M. mozambicus*, *M. sagamiensis* and *M. velutinus*. The relationship among these four species suggested by the 16S rRNA data set was different from that suggested by COI data set. In the three COI gene trees, *M. sagamiensis* and *M. velutinus* appeared to be more closely related to each other, supported by BP values of 98, 60 and 82% in NJ, ML and MP trees, respectively. These two species were then more related to *M. andamanicus* than to *M. mozambicus*, this relationship was supported with BP ≥ 71% in the NJ and MP trees (Figures 3.5 and 3.7) and has weak BP of < 50% supported in the ML tree (Figure 3.6).

3.3.7 Phylogenetic analysis based on combined data set

The sequence length was 1718 bp after combining sequences of 16S rRNA and COI genes. The partition homogeneity test resulted in a value of $p = 0.70$ for the combined data set. The null hypothesis of this test is the sequences of 16S rRNA and COI genes show homogeneity in the distribution of phylogenetic information. Since the *p*-value of partition homogeneity test was lager than 0.05, the null hypothesis could not be rejected, suggesting there was no significant incongruence within the combined data set. Phylogenetic analyses, therefore, could be carried out based on the combined data set. The best-fitting model suggested by Modeltest 3.7 for the combined data set was Tamura-Nei model (Tamura and Nei, 1993) with a proportion of invariable sites and with a gamma distribution (TrN + I + G; Table 3.9). The PTP test confirmed that tree lengths of the combined data set were highly significant (P = 0.01).

The topologies of NJ, ML and MP phylogenetic trees generated based on the combined data set (Figures 3.8 to 3.10) were identical to those generated based on COI data set. In most cases, the bootstraps values supporting the clades in the phylogenetic trees based on combined data set were higher than those based on COI data set. NJ and ML analyses based on combined data set (Figures 3.8 and 3.9)

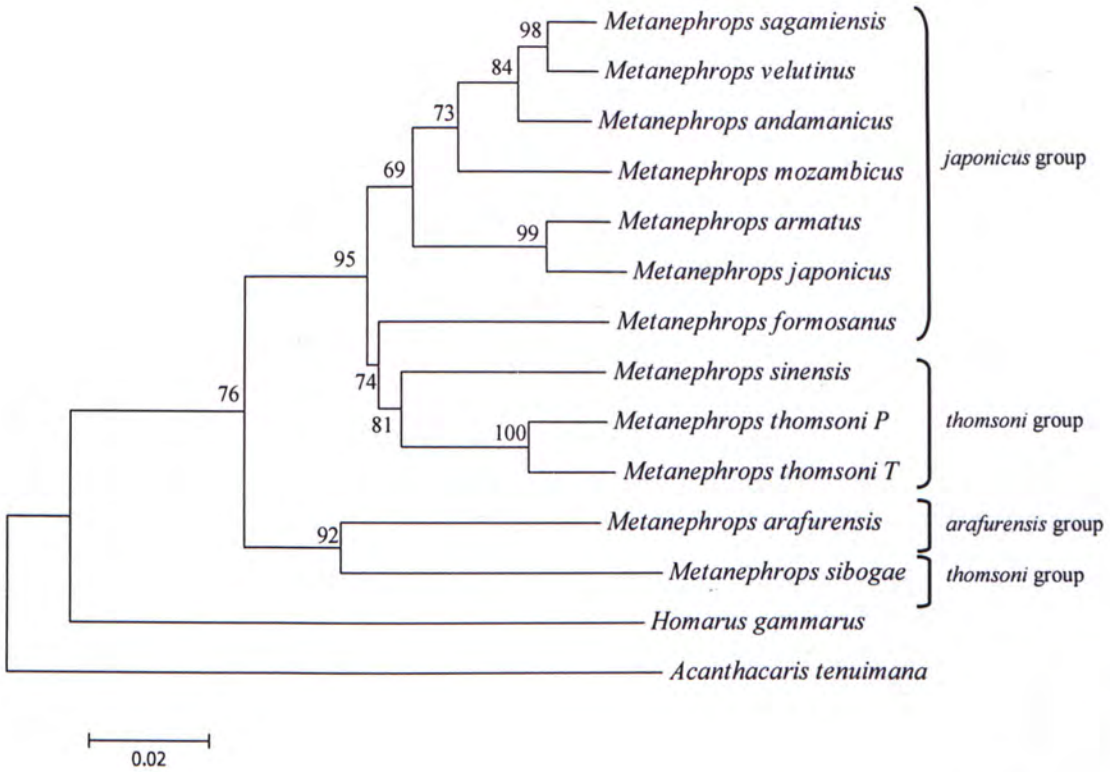

Figure 3.8 Neighbor-joining tree derived from combined data set. Numbers above branches are bootstrap values (1000 replicates). Bootstrap values below 50% are not shown.

Metanephrops thomsoni P: *M. thomsoni* collected from the Philippines

Metanephrops thomsoni T: *M. thomsoni* collected from Taiwan

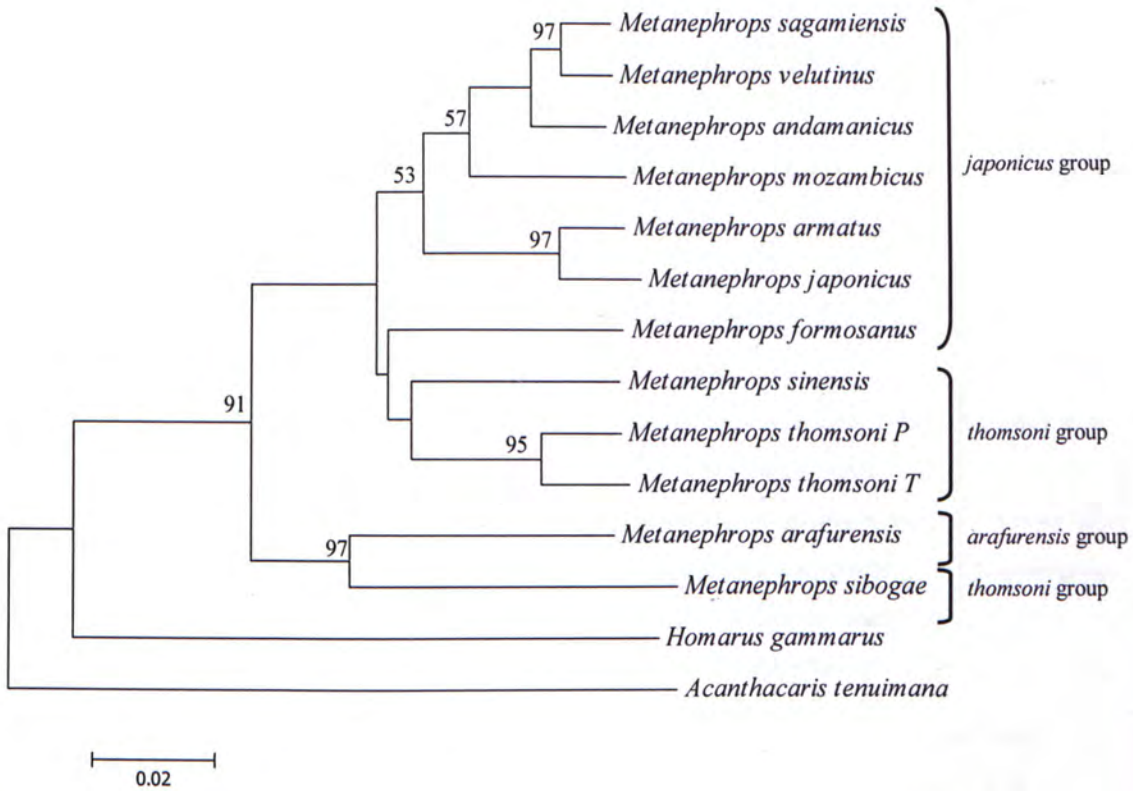

Figure 3.9 Maximum likelihood tree derived from combined data set. Numbers above branches are bootstrap values (500 replicates). Bootstrap values below 50% are not shown.

Metanephrops thomsoni P: *M. thomsoni* collected from the Philippines
Metanephrops thomsoni T: *M. thomsoni* collected from Taiwan

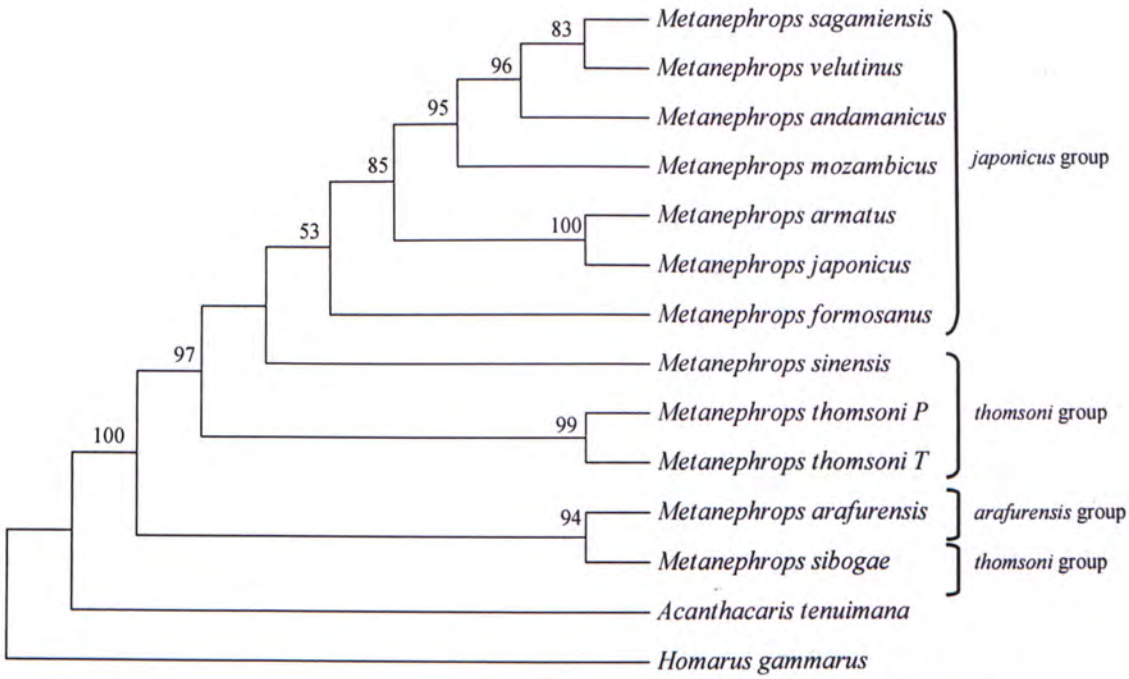

Figure 3.10 Maximum parsimony tree derived from combined data set. Numbers above branches are bootstrap values (1000 replicates). Bootstrap values below 50% are not shown.

Metanephrops thomsoni P: *M. thomsoni* collected from the Philippines
Metanephrops thomsoni T: *M. thomsoni* collected from Taiwan

constructed the same tree topology, whilst the topology inferred by MP analysis (Figure 3.10) was similar to those of the NJ and ML analyses. The monophyletic relationship of genus *Metanephrops* from the two outgroups, *A. tenuimana* and *H. gammarus*, was supported with BP ≥ 76% in all analyses.

Similar to the results based on COI gene data set, the combined data set also indicated there were two major clades of *Metanephrops*. The clade containing *M. arafurensis* (*arafurensis* group) and *M. sibogae* (*thomsoni* group) was supported by high bootstrap values (BP ≥ 92%) in all analyses. Another major clade contains the remaining species (i.e. all species from *japonicus* group, *M. sinensis* and *M. thomsoni*), was supported by BP values of 95, <50 and 97% in NJ, ML and MP trees, respectively.

Both the 16S rRNA and COI data sets suggested that the two specimens of *M. thomsoni* (*thomsoni* group) collected from different localities were closely related to each other. This was also supported by high confidence levels (BP ≥ 95%) in all trees inferred from the combined data set. Both NJ and ML trees indicated that *M thomsoni* was more related to *M. sinensis* (supported by BP of 81 and < 50% in NJ and ML trees, respectively) than to *M. formosanus* (supported by BP of 74 and <

50% in NJ and ML trees, respectively). On the contrary, the MP tree showed that *M. sinensis* was closely related to all species from the *japonicus* group which was supported with a weak BP value of < 50%.

The relationships among species from *japonicus* group suggested by the combined data set were identical to that of suggested by COI data set, and the BP supported in most cases were higher in those combined analyses. Except *M. formosanus*, all species from the *japonicus* group clustered together with 69, 53 and 85% bootstrap values supported in NJ, ML and MP trees, respectively. Similar to the relationships suggested by the 16S rRNA and COI data sets, the combined data set also suggested that other than *M. formosanus*, the species from *japonicus* group were divided into two major clades. The clade consisting of *M. armatus* and *M. japonicus* was supported by high confidence levels (BP ≥ 97%) in all analyses, while another clade with *M. andamanicus*, *M. mozambicus*, *M. sagamiensis* and *M. velutinus* was supported by BP of 73, 57 and 95% in NJ, ML and MP trees, respectively. Among these four species, *M. sagamiensis* and *M. velutinus* were closely related to each other and they were more related to *M. andamanicus* than to *M. mozambicus*, and the BP support these relationships in most cases were high (BP ≥ 83%).

3.4 Discussion

3.4.1 Interspecific genetic divergence

In the present study, the sequence divergences of conspecific individuals (*M. andamanicus*, *M. australiensis*, *M. challengeri*, *M. japonicus*, *M. sagamiensis* and *M. sibogae*) collected at the same locality are always lower than 1%. The sequence divergences of specimens of *M. neptunus* and *M. thomsoni* collected at different localities were larger than 1% (Tables 3.6 and 3.7). 16S rRNA gene divergences between the two conspecific individuals of *M. neptunus* and *M. thomsoni* are both 0.0124 substitutions per site (subst./site) while the COI gene sequence divergence between the two conspecific individuals of *M. thomsoni* is 0.0361 subst./site. Lefébure *et al.* (2006) recently proposed a threshold for crustacean species delimitation. If COI divergence of two monophyletic taxa is higher than 0.16 subst./site, and it is possible that they are two different species. The level of divergence between the two conspecific *M. thomsoni* is smaller than the proposed threshold by Lefébure *et al.* (2006). Hence, the two *M. thomsoni* specimens from the Philippines and Taiwan are possibly to be two different species. Only 16S rRNA sequence divergence is available between the two conspecific *M. neptunus*, but the level of sequence divergence in 16S rRNA gene between *M. neptunus* and *M. thomsoni* is the same. There is no 16S threshold suggested for species delimitation. It

can be expected that the level of sequence divergence of COI between the two *M. neptunus* specimen is also similar to that of the *M. thomsoni*. Therefore, there is a strong possibility that the two *M. neptunus* specimens each from Indonesia and Pratas belong to the same species.

3.4.2 Monophyly of the four species groups

Since the erection of *Metanephrops* (Jenkins, 1972), there have been no controversy about the four traditional morphological-based groupings of *Metanephrops* and this grouping has been widely adopted in various studies (e.g. Chan and Yu, 1987; 1988; 1991; Chan, 1997). The phylogenetic status of this grouping is evaluated in the present study. The present study only supports the monophyly of the *binghami* and *japonicus* groups. In all phylogenetic analyses based on 16S rRNA gene sequence (Figures 3.2 to 3.4), the monophyly of *binghami* group is strongly supported by 100% bootstrap values. Moreover, except *M. formosanus*, all members of the *japonicus* group in all phylogenetic trees in the present study show that species in *japonicus* group cluster together, especially those based on the combined data set, with bootstrap values of 53% or above support this relationship.

The traditional *arafurensis* and *thomsoni* groups (Jenkins, 1972) are not supported in the phylogenetic analyses in the present study. Results from this study indicate that species from *arafurensis* and *thomsoni* groups do not cluster together. The present study, therefore, do not support the monophyletic origin of *arafurensis* or *thomsoni* groups.

Since the validity of *arafurensis* and *thomsoni* groups is not supported in the study, regrouping of the four species groups is needed. Based on the phylogenetic trees in the present study, species from *Metanephrops* should be divided into five species groups instead of four (Table 3.10). Relatively high bootstrap values in the 16S rRNA phylogenetic trees (Figures 3.2 to 3.4) support the monophyly of *M. challengeri* and *M. neptunus*, suggesting these two species should belong to the same group. In addition, the monophyly of *M. binghami* and *M. rubellus* in the 16S rRNA phylogenetic trees also suggest that these two species should be regarded as a second group. The third species group contains *M. arafurensis*, *M. australiensis* and *M. sibogae*. These three species always cluster together in the 16S rRNA phylogenetic trees. In addition, the monophyletic relationship of *M. arafurensis* and *M. sibogae* in the COI and combined phylogenetic trees (Figures 3.5 to 3.10) also provides support

Table 3.10 The five new species groups of *Metanephrops*

Species group	Group member
One	*M. challengeri*
	M. neptunus
Two	*M. binghami*
	M. rubellus
Three	*M. arafurensis*
	M. australiensis
	M. sibogae
Four	*M. andamanicus*
	M. armatus
	M. japonicus
	M. mozambicus
	M. sagamiensis
	M. velutinus
Five	*M. formosanus*
	M. sinensis
	M. thomsoni

to the grouping of these two species. The validity of *japonicus* group is supported in the present study when *M. formosanus* is not included, this group should represent the fourth group. In most of the cases, the relationship of *M. formosanus*, *M. sinensis* and *M. thomsoni* with the other species cannot be resolved. These three species spear to be genetically intermediate between the *japonicus* group and the third species group. Therefore, these three genetically intermediate species should be included as the fifth group.

3.4.3 Phylogenetic relationship in *Metanephrops*

The phylogenetic trees inferred from 16S rRNA gene sequences (Figures 3.2 to 3.4) suggest that *M. challengeri* (*thomsoni* group) and *M. neptunus* (*arafurensis* group) cluster together at the basal position in the trees. The sequence divergence between these two species and the outgroup taxa is the lowest among values between species in *Metanephrops* and the outgroup taxa (Table 3.6). This indicates that the two species are the primitive members in *Metanephrops*. Jenkins (1972) suggested that *arafurensis* group is one of the oldest groups in *Metanephrops* and *M. neptunus* is the most primitive species among members in *arafurensis* group. Chan (1997) stated that the general appearance of *M. neptunus* is different from all the other species in *Metanephrops*. The result from a morphology-based cladistic analysis also

indicates that *M. challengeri* is the most primitive extant species (Tshudy *et al.*, unpublished). Results from this study, hence, provide evidence further support the suggestions based on morphology.

The present study does not support Jenkins' (1972) suggestion that *thomsoni* group derived from the ancestor of *M. australiensis*. Phylogenetic trees in this study show that some *thomsoni* species (*M. challengeri* and *M. sibogae*) are more related to *arafurensis* group while the other species (*M. sinensis* and *M. thomsoni*) are more related to *japonicus* group. Since the present study does not support the monophyletic origin of *thomsoni* group, it is not possible to suggest that *thomsoni* group is derived from any species in *arafurensis* group.

Jenkins (1972) proposed that there is a common ancestor between *binghami* and *japonicus* groups. Results in this study show that species from *binghami* group do not share a direct common ancestor with *japonicus* group. In contrast, the *binghami* group is more related to species from *arafurensis* and *thomsoni* groups. Although phylogenetic trees (Figures 3.2 to 3.4) in this study support the monophyletic relationship of *binghami* group, the relationship between *binghami* and *japonicus* groups suggested by Jenkins (1972) is not supported.

Even though molecular data from the present study do not support the monophyly of *arafurensis* or *thomsoni* groups, some of the relationships among the species in these two groups suggested by morphology are consistent with the results from the present study. Several authors noted that *M. australiensis* is morphologically most similar to *M. arafurensis* (Jenkins, 1972; Chan and Yu, 1987; Holthuis, 1991). Molecular data in the present study showed the close relationship between these two species. Bootstrap values of > 81% in the three 16S rRNA gene trees (Figures 3.2 to 3.4) support that *M. australiensis* and *M. arafurensis* are sister taxa. It has also been suggested that *M. sibogae* is closely related to *M. boschmai* (Chan, 1997). In addition, 16S rRNA, COI and the combined data set support that *M. sibogae* is closely related to *M. arafurensis*. However, the relationship between theses species and *M. boschmai* cannot be resolved as *M. boschmai* sequences are not available in this study.

The present study suggests three different relationships between *M. thomsoni*, *M. sinensis* and species in *japonicus* group in the analyses. The NJ and ML trees inferred from COI gene and the combined data set (Figures 3.5 to 3.6 and 3.8 to 3.9) suggest that *M. formosanus* is more related to *M. thomsoni* and *M. sinensis* as compared to *japonicus* group. However, the NJ and ML trees based on 16S rRNA

gene (Figures 3.2 to 3.4) and MP trees based on COI gene and combined data set (Figures 3.7 and 3.10), suggest a different relationship that *M. sinensis* is more closely related to *japonicus* group than to *M. thomsoni.* In addition, the MP tree based on 16S rRNA gene (Figure 3.4) suggests a third relationship that *M. sinensis* is closely related to *japonicus* group while *M. thomsoni* is more closely related to *M. sibogae*. The phylogenetic relationship of *M. formosanus*, *M. thomsoni* and *M. sinensis*, therefore, could not be resolved in this study. Other gene regions from mitochondrial or nuclear DNA are required for elucidating the relationship of these two species in *Metanephrops*.

It has been proposed that *M. formosanus* is more related to and belongs to the *japonicus* group based on current morphological definitions of the species groups (Chan and Yu, 1991). Chu *et al.* (1990) investigated the genetic relatedness of the three species of *Metanephrops* from Taiwan based on electrophoretic analysis of isozymes. The results suggested that the '*M. japonicus var.*' [later described as *M. armatus* (Chan and Yu, 1991)] and *M. formosanus* are closely related. Some of the phylogenetic trees in the present study show that *M. formosanus* always clusters to other species in *japonicus* group, and this is consistent with the previous suggestion based on morphology and electrophoretic analysis that *M. formosanus* is a member

of *japonicus* group. However, other phylogenetic trees in the present study showed that *M. formosanus* is more related to *M. thomsoni* and *M. sinensis*. In addition, Jenkins (1972) suggested that the *japonicus* and *thomsoni* groups are distantly related. On the contrary, this contradicts with the intermediate status of *M. formosanus* between *japonicus* and *thomsoni* groups as suggested by their morphology (Chan and Yu, 1987; 1988; 1991). Chan and Yu (1991) noted the morphology of *M. formosanus* is unique in *japonicus* group (e.g. the big chelae armed with large spines) and it is morphologically intermediate between *japonicus* and *thomsoni* groups (Chan and Yu, 1987; 1988). The close relationship of *M. formosanus* with *japonicus* groups and some species in *thomsoni* group is in agreement with the intermediate status of *M. formosanus* between *japonicus* and *thomsoni* groups based on morphology. Future study, therefore, should be carried out to elucidate the relationship of *M. formosanus* with *japonicus* group based on other gene regions from mitochondrial and nuclear DNA.

Jenkins (1972) proposed that the *arafurensis* and *japonicus* groups are the oldest among modern species. The present study does not support the monophyly of the *arafurensis* group, but do support one of the species in *arafurensis* group (*M. neptunus*) at the basal position in the phylogenetic trees. Moreover, species in

japonicus group always in the derived position in the phylogenetic trees. The primitive status of *arafurensis* and *japonicus* group are, therefore, not supported in the present study.

Results from the molecular data in this study are consistent with some of the relationship among *japonicus* group suggested based on morphology. Chan and Yu (1991) made a comprehensive taxonomic account of the species in *japonicus* group based on morphology. They described that the general appearance of *M. armatus* is quite similar to that of *M. japonicus*. Thus, it is believed that these two species are closely related. The close relationship between these two species is strongly supported in this study. All phylogenetic trees (BP $\geq$ 85%) show that these two species are sister taxa.

Molecular data in the present study partly support the relationship between some species in *japonicus* group as suggested by morphology. COI and the combined data set (Figures 3.5 to 3.10) suggest that *M. sagamiensis* and *M. velutinus* are sister taxa and they are more related to *M. andamanicus* than to *M. mozambicus*. However, this relationship is not consistent with the similarity in general appearance between *M. sagamiensis* and *M. andamanicus* observed by De Man (1916). On the other hand,

Chan and Yu (1991) stated that *M. andamanicus* is more closely related to *M. mozambicus* based on morphology.

3.4.4 Evolutionary history of *Metanephrops*

Jenkins (1972) proposed that the Indo-West Pacific region is where *Metanephrops* originated. Chan (1997) also supported this and further confined the area to the Indo-Malay region. In contrast, Feldmann and Tshudy (1989) suggested that *Metanephrops* evolved in or near to the Antarctica. Moreover, Jenkins (1972) has proposed two migration routes of *binghami* group: the ancestor of *binghami* group arrived Atlantic (1) via southern Africa or (2) migrated though the Tethys Sea during or before the Lower Miocene.

Based on the phylogenetic trees inferred from 16S rRNA gene sequences (Figures 3.2 to 3.4), *M. challengeri* and *M. neptunus* are placed at the basal position in the trees. The distribution of *M. challengeri* is endemic to New Zealand while *M. neptunus* is widely distributed from the South China Sea to western Australia (Chan, 1997). Moreover, *M. challengeri* is the most southern species among species in *Metanephrops*. The basal position of *M. challengeri* in phylogenetic trees also provides support to the suggestion by Feldmann and Tshudy (1989) that origin of

Metanephrops is near Antarctica.

Since there was no calibrated rate of molecular evolution for *Metanephrops*, the divergence time estimated in this study is based on the calibrated divergence rates of 0.9% per million years for the 16S rRNA gene in *Uca* (Sturmbauer *et al.*, 1996). The divergence time between the *binghami* group and *M. challengeri* is estimated to 15.7 million years ago (the Lower Miocene). The divergence time estimated from 16S rRNA gene is consistent with that of the time suggested in Jenkins (1972). Ancestor of the *binghami* group, as suggest in the present study, dispersed to Atlantic around the Lower Miocene. Moreover, the monophyletic relationship of *binghami* group indicates that there was only one invasion of this group to the Atlantic. However, results from the present study could not provide any information to discriminate the two possible routes of *binghami* group.

Based on the phylogenetic trees inferred in the present study, it can be speculated that *Metanephrops* evolved near the Antarctic region. The ancestor of *M. neptunus* arrived western Australia and then dispersed northward to the South China Sea. After the emergence of the *binghami* group, the common ancestor of the remaining extant species diversified at Indo-West Pacific region.

Chapter 4

Molecular Identification of Nephropidae

4.1 Introduction

The decline in biodiversity in natural environments becomes a hot issue in modern human society. Many conservation works in saving biodiversity have been done. Biologists, however, during conservation works face a problem referred to as taxonomic impediments. For example, species identification is difficult due to the lack of taxonomists. In addition, taxonomic literatures are sometimes difficult to access and species identification is often difficult because of only parts of the specimen could are available. To overcome these impediments, DNA barcodes have been suggested to aid species identification and traditional taxonomy. Such molecular taxonomy has already been set up for groups which are difficult to identify based solely on morphological parameters, such as bacteria, virus and fungi for a longtime.

Nephropidae is a diverse clawed lobster family. There are 52 extant species in 13 genera in Nephropidae. It has been suggested that the claw morphology was strongly and direct affected by environment pressures (Tshudy and Sorhannus, 2000). Field specimens only with claws, therefore, could not be used to identify clawed

lobster species. Molecular technique (RAPD) has been developed to discriminate some of the species in Nephropidae (Hughes and Beaumont, 2004). However, this method is complicated and sometimes is found unreliable. The present study aims to evaluate the ability and feasibility of species discrimination in Nephropidae by using sequences from two mitochondrial genes, the large subunit ribosomal rRNA (16S rRNA) and cytochrome *c* oxidase subunit I (COI) genes.

4.2 Materials and methods

4.2.1 Species studied and sample collection

Specimens were stored in collections from museums, the National Taiwan Ocean University in Keelung, Taiwan, Muséum national d'Histoire naturelle in Paris, France and Nationaal Natuurhistorisch Museum in Leiden, the Netherlands. Specimens were preserved in 70% ethanol when received. Some of the specimens had been preserved in formalin during the storage in museums. Table 4.1 shows the specimens collection localities and the present depository storage.

Table 4.1 Sampling localities and present depository of species sequenced in this study

Species	No. of individuals	Sampling locality (Present depository*)
Acanthacaris tenuimana	2	Solomon Island (MNHNP)
Enoplometopus crosnieri#	1	Taiwan (NTOU)
Enoplometopus daumi#	1	Singapore aquarium shop (NTOU)
Enoplometopus debulis#	1	Singapore aquarium shop (NTOU)
Enoplometopus occidentalis#	1	Taiwan (NTOU)
Eunephrops cadenasi	1	Guadeloupe (MNHNP)
Eunephrops manningi	1	Guadeloupe (MNHNP)
Homarus gammarus	1	Paris supermarket (NTOU)
Metanephrops arafurensis	1	Indonesia (MNHNP)
Metanephrops binghami	1	NW coast of Panama, Mosquito Gulf (NNML)
Metanephrops formosanus	1	Taiwan (NTOU)
Metanephrops japonicus	1	Sagami Bay, Japan (NTOU)
Metanephrops thomsoni	1	Philippines (NTOU)
Nephropides caribaeus	1	Guadeloupe (MNHNP)
Nephrops norvegicus	2	Paris supermarket (MNHNP)
Nephropsis serrata	2	Taiwan (NTOU)
Thaumastocheles dochmiodon	1	Taiwan (NTOU)
Thaumastocheles japonicus	1	Taiwan (NTOU)
Thymopides grobovi	2	Kerguelen Island (MNHNP)

Test taxa related to Nephropidae

* MNHNP: Muséum national d'Histoire naturelle in Paris, France
NNML: Nationaal Natuurhistorisch Museum in Leiden, the Netherlands
NTOU: National Taiwan Ocean University in Keelung, Taiwan

4.2.2 DNA extraction

DNA extraction procedures were described in Section 3.2.2.

4.2.3 Amplification of genes

Partial mitochondrial 16S rRNA and COI genes regions were amplified from total DNA by polymerase chain reaction (PCR) (Saiki *et al.*, 1988).The primers pair 16S ar (Simon *et al.*, 1994) and 16S 1472 (Crandall and Fitzpatrick, 1996) was used for amplifying the partial 16S rRNA gene (Table 4.2) and the expected PCR product size was approximately 580 bp. The primer pair LCO 1490 and HCO 2198 (Folmer *et al.*, 1994; Table 4.2) was used to amplify the 5' end of COI gene segment and the expected PCR product size was 700 bp.

Table 4.2 Primer sequences used in the amplification and sequencing in this study

Primer name	Primer sequence 5' 3'
16S ar	CGCCTGTTTATCAAAAACAT
16S 1472	AGATAGAAACCAACCTGG
LCO 1490	GGTCAACAAATCATAAAGATATTGG
HCO 2198	TAAACTTCAGGGTGACCAAAAAATCA

4.2.4 PCR profiles for mitochondrial genes

The procedures for gene amplification were described in Section 3.2.3.

4.2.5 Nucleotide sequencing

Nucleotide sequencing was performed as described in Section 3.2.4.

4.2.6 Purification of asymmetric PCR products

Procedures for purification of PCR products were described in Section 3.2.4.2.

4.2.7 Sequence alignment

Nucleotide sequence from each individual was inspected with the aid of ABI Sequence Editor 1.0.3 (Applied Biosystems) and confirmed with reference to the data from both strands. Any ambiguous bases were noted and designated as unknown by ABI Sequence Editor 1.0.3.

4.2.8 Cluster analysis

Two gene profiles, 16S rRNA and COI, were constructed based on distance BIO neighbor-joining (NJ) method using MEGA version 3.1 (Kumar *et al.*, 2004). In NJ analysis, sequence divergences were calculated using Kimura-two-parameter (K2P) model (Kimura, 1980).

4.2.9 Graphical summary of species similarity

In order to allow the genetically intermediate taxa to remain spatially intermediate instead of forcing them to cluster with other taxa as in tree construction (Lessa, 1990), matrix of pairwise K2P estimates of sequence divergence between species of Nephropidae studied was constructed. Graphical summary of the species similarity based on multidimensional scaling (MDS) analysis was constructed using SPSS 13.0 for Windows based on the sequence divergence matrix.

4.2.10 Testing of molecular identification system in Nephropidae

In order to evaluate the ability of taxa discrimination in Nephropidae at different taxonomic levels, three kinds of test taxa were included. First, other individuals belonging to the same species already included in the profile were added into the profile (Table 4.1). Second, four species from family Enoplometopidae (Table 4.1) which is closely related to Nephropidae (Ahyong and O'Meally, 2004) were added into the test profile. Third, two freshwater crayfishes species that are distantly related to Nephropidae were added into the test profile (Table 4.3). NJ and MDS analyses were re-run based on the sequences from Nephropidae together with those sequences from test taxa.

Table 4.3 List of test taxa related to Nephropidae being included in the test profiles

Family	Species	GenBank accession number	
		16S rRNA	COI
Astacidae	*Astacus astacus*	AF235983	AY151515
Parastacidae	*Cherax quadricarinatus*	DQ006552	DQ006294

4.3 Results

4.3.1 PCR products and sequence alignments of 16S rRNA and COI genes

No 16S rRNA PCR product could be amplified from the specimen of *Enoplometopus occidentalis*. For other species, 570 bp of 16S rRNA PCR products were amplified. The segment length of COI gene PCR products amplified from all specimens was 700 bp. There was no COI PCR product, however, could be amplified from the specimen of *Metanephrops binghami*.

After aligning the 16S rRNA gene sequences, the length of the final aligned and truncated sequences (including gaps) from the 15 nephropid species and the nine test taxa was 421 bp (Appendix 3). The length of aligned and truncated sequences of COI gene from the 14 nephropid species and the 10 test taxa was 568 bp (Appendix 4).

4.3.2 Species identification for clawed lobsters

4.3.2.1 16S rRNA profile

Each of the 15 nephropid species being examined in the 16S rRNA profile possessed a unique sequence except *Thaumastocheles dochmiodon* and *T. japonicus*, in which the sequences were identical. For the other Nephropidae species studied, the interspecific sequence divergence ranged from 2.3% between *Metanephrops*

japonicus and *M. thomsoni* to 20.3% between *M. binghami* and *Nephropsis serrata*, with a mean of 13.0%. The intraspecific sequence divergence among conspecific individuals of Nephropidae (*Acanthacaris tenuimana, Nephrops norvegicus, Nephropsis serrata* and *Thymopides grobovi*) was ranging from 0 to 1.5%, with a mean of 0.3%.

Taxa belonged to the same genes were clustered together in the NJ tree based on 16S rRNA sequences (Figure 4.1). For genera with one representative species, *Homarus gammarus* and *Nephrops norvegicus* were more closely related to each other than to the two species from *Thaumastocheles*. *Acanthacaris tenuimana* and *Nephropsis serrata* were grouped. The two species of *Eunephrops* were closely related to each other and were more related to *Nephropides caribaeus* than to *Thymopides grobovi*. The relationship inferred from the MDS analysis based on 16S rRNA gene sequences (Figures 4.2) was similar to the relationship suggested by NJ analysis (Figure 4.1). The two species in *Thaumastocheles* were placed in the same coordinates, *Homarus gammarus* and *Nephrops norvegicus* were more closely related to each other than to the two *Thaumastocheles* species. In addition, *Nephropsis serrata* was the most distantly related to the other species in Nephropidae studied. However, some relationships shown in the MDS analysis (Figure 4.2) are

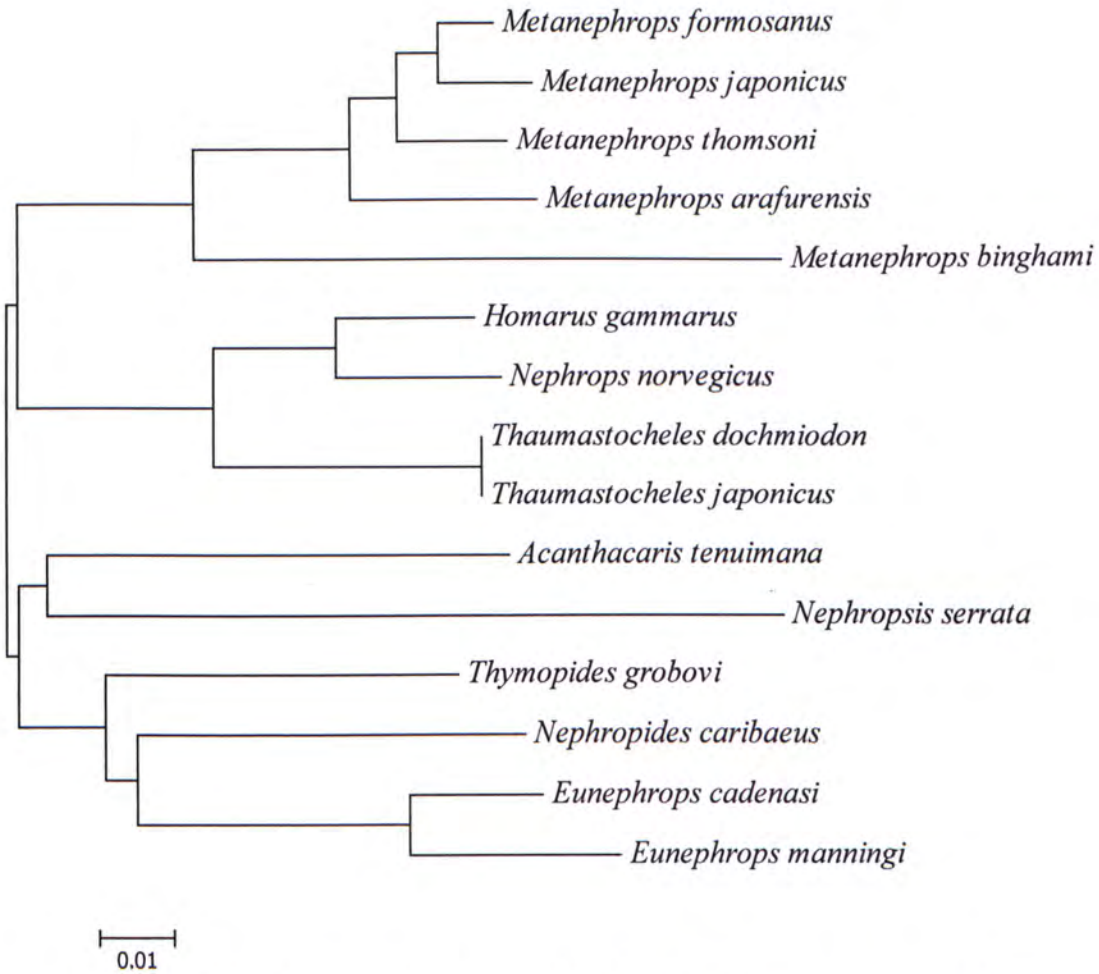

Figure 4.1 Neighbor-joining tree of mitochondrial 16S rRNA gene sequences from 15 species of Nephropidae.

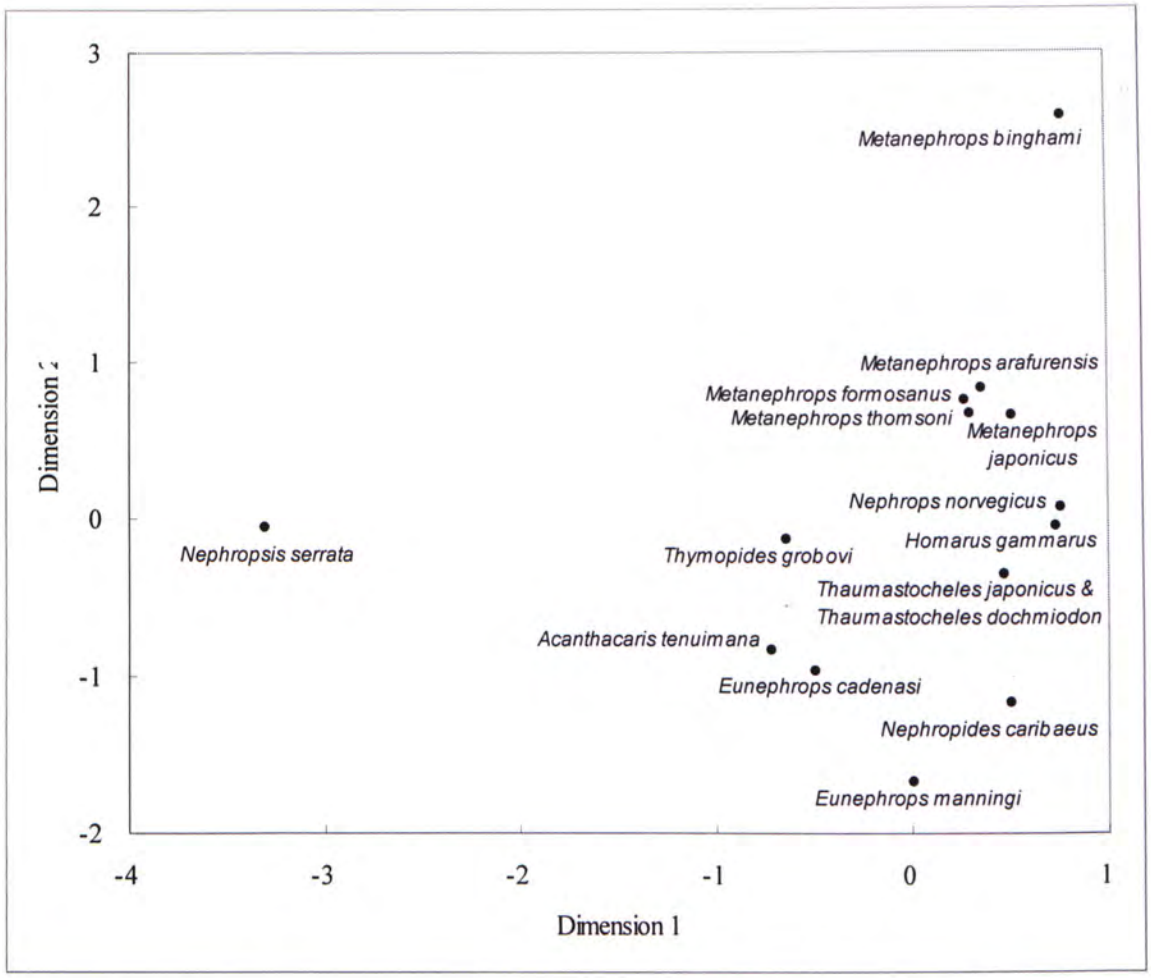

Figure 4.2 Multidimensional scaling of genetic distance based on mitochondrial 16S rRNA gene sequences from 15 species of Nephropidae.

not consistent with that of the NJ analysis (Figure 4.1). *M. binghami* was distantly related to other species of *Metanephrops* in the MDS analysis, and the two species in *Eunephrops* were not closely related to each other. *Eunephrops manningi* was more related to *Nephropides caribaeus* than to *Eunephrops cadenasi.*

The re-run NJ analysis based on 16S rRNA gene sequences (Figure 4.3) included 15 species of Nephropidae and the nine test taxa. The topology of the re-run NJ tree (Figure 4.3) is the same as the original one (Figure 4.1), except that *Acanthacaris tenuimana* and *Nephropsis serrata* did not cluster together in the re-run NJ analysis (Figure 4.3). The re-run NJ analysis showed that all conspecific test taxa were assigned to their corresponding species correctly. All species from Nephropidae were closely related and respect to the test taxa which do not belong to but related to Nephropidae. The three species from *Enoplometopus* clustered together and were more related to species of Nephropidae than to *Astacus astacus* and *Cherax quadricarinatus*.

The re-run MDS analysis based on 16S rRNA gene sequences (Figure 4.4) included nine test taxa and the 15 species of Nephropidae. The relationship among

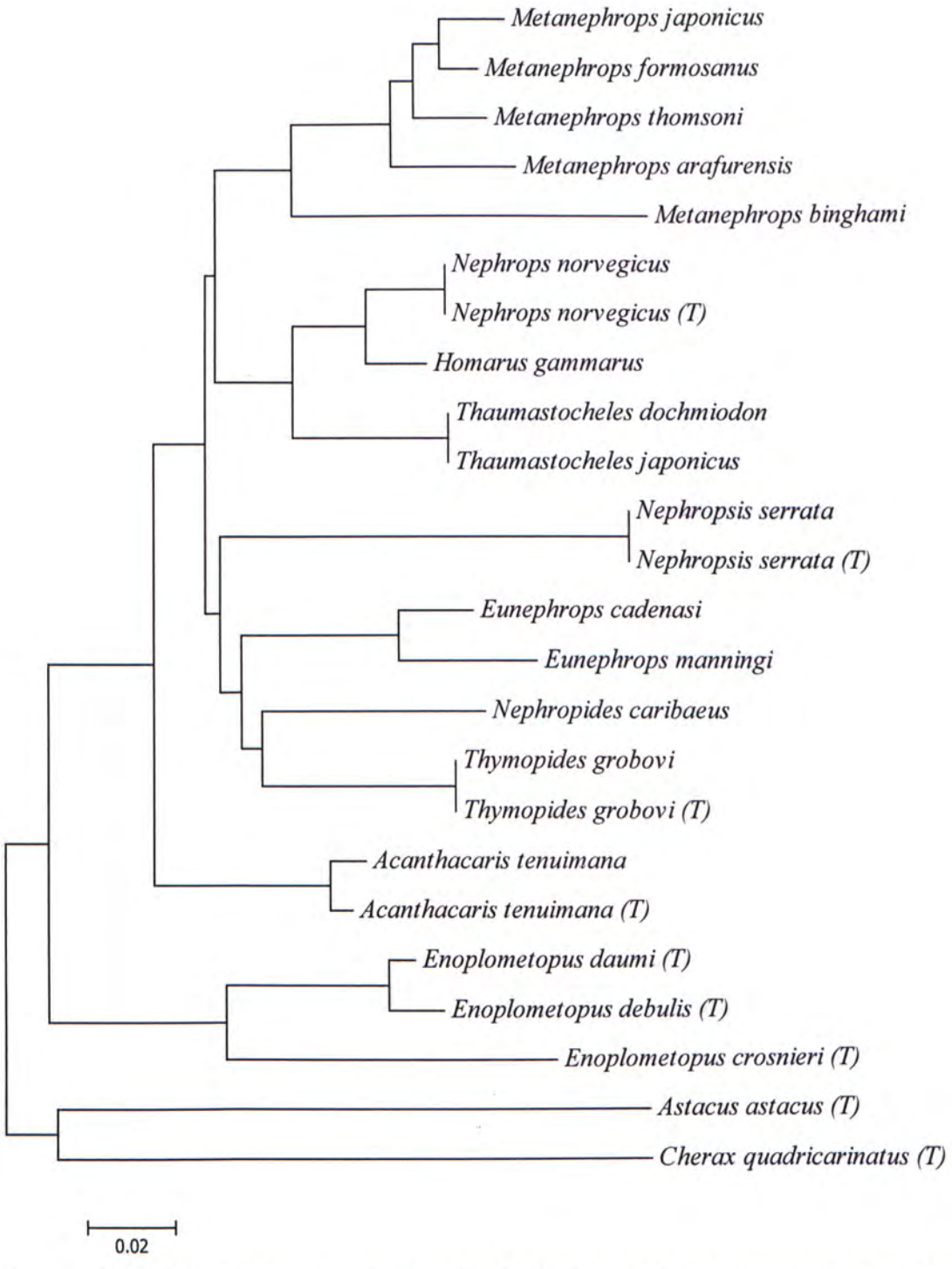

Figure 4.3 Neighbor-joining tree of mitochondrial 16S rRNA gene sequences from 15 species of Nephropidae and the nine test taxa. Species name followed by *(T)* are test taxa.

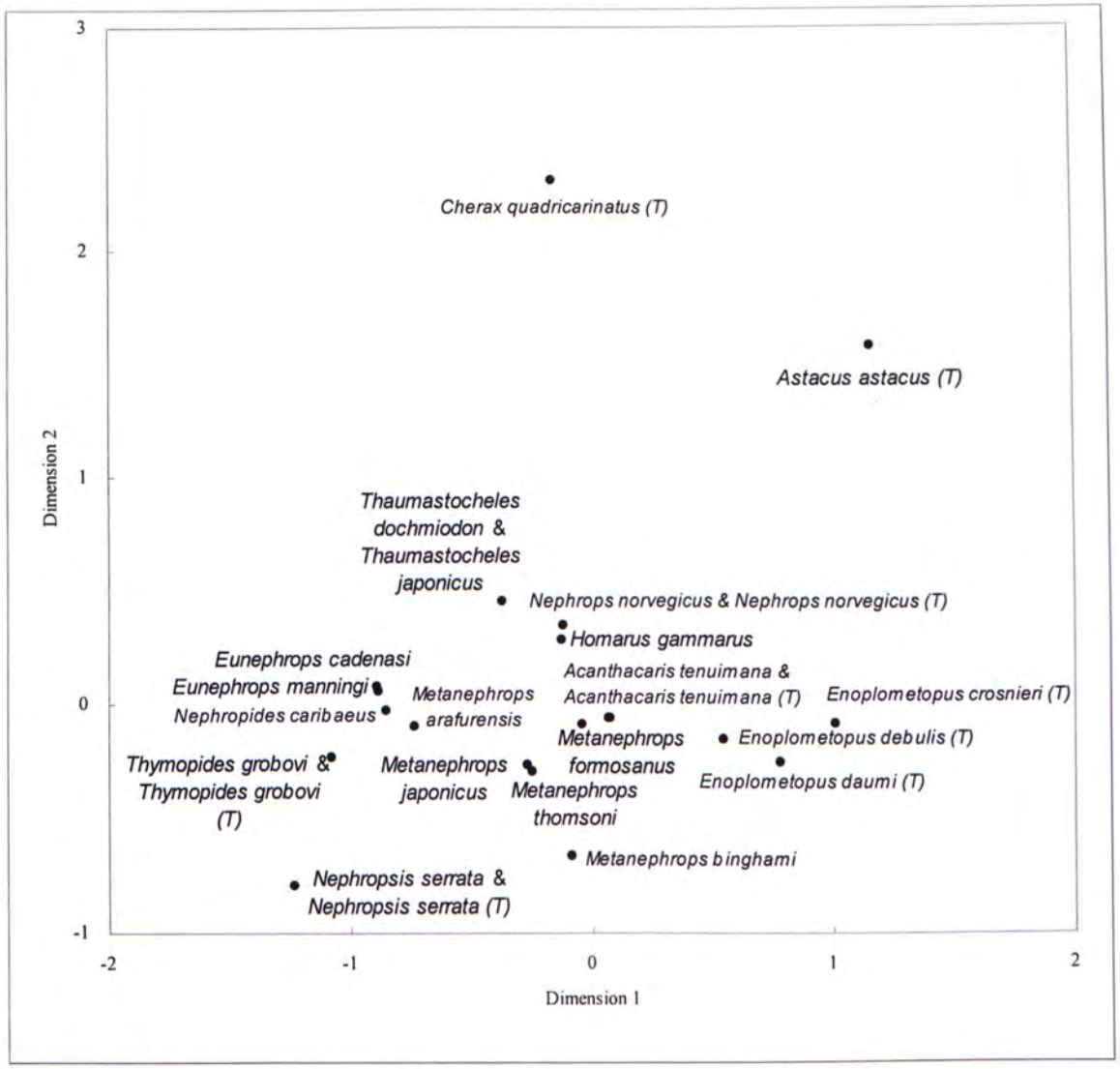

Figure 4.4 Multidimensional scaling of genetic distance based on mitochondrial 16S rRNA gene sequences from 15 species of Nephropidae and the 9 test taxa. Species name followed by *(T)* are test taxa.

species of Nephropidae inferred from the re-run 16S MDS analysis (Figure 4.4) were slightly different from that derived from the original 16S MDS analysis (Figure 4.2). On the other hand, the re-run 16S MDS analysis (Figure 4.4) was partly consistent with the relationships shown in the re-run 16S NJ analysis (Figure 4.3). All conspecific test taxa were at the same coordinates as their corresponding species. Two species of *Metanephrops* (*M. japonicus* and *M. thomsoni*) were closely related to each other while the remaining three species (*M. arafurensis*, *M. binghami* and *M. formosanus*) were distantly related to the former two species. In addition, *M. arafurensis* was closely related to *Nephropides caribaeus* and *M. formosanus* was closely related to *Acanthacaris tenuimana*. The two *Eunephrops* species were closely related to each other and were related to *Nephropides caribaeus* than to *Thymopides grobovi*. *Nephropsis serrata* was, in addition, related to *Thymopides grobovi* distantly. The relationships between *Homarus gammarus* and *Nephrops norvegicus* and the two species from *Thaumastocheles* shown in the 16S re-run MDS analysis (Figure 4.4) was consistent with that of the 16S re-run NJ analysis (Figure 4.3), the former two species were closely related to each other than to the two *Thaumastocheles* species. Besides, the two *Thaumastocheles* species were placed at the same coordinates in the MDS analysis (Figure 4.4). *Acanthacaris tenuimana* was more closely related to species of Nephropidae and in a position intermediate between

species of Nephropidae and the three species of *Enoplometopus*. The three test species of *Enoplometopus* were closely related to each other than to those species from Nephropidae. Moreover, the two test taxa that are distant but related to Nephropidae, *Astacus astacus* and *Cherax quadricarinatus*, were neither related nor grouped to the species in Nephropidae.

4.3.2.2 COI profile

Fourteen species from family Nephropidae were included in the COI profile. Apart from *Thaumastocheles dochmiodon* and *T. japonicus*, different species being examined possessed different COI gene sequences. Except *T. dochmiodon* and *T. japonicus*, sequence divergence between different species from Nephropidae ranged from 10.1% between *M. formosanus* and *M. thomsoni* to 27.7% between *Acanthacaris tenuimana* and *Nephropsis serrata*, with a mean of 22.7%. The intraspecific sequence divergence among conspecific individuals of Nephropidae (*Acanthacaris tenuimana*, *Nephrops norvegicus*, *Nephropsis serrata* and *Thymopides grobovi*), was ranging from 0 to 3.4%, with a mean of 0.9%.

The topology of NJ tree based on COI gene sequences (Figure 4.5) was similar to that of the 16S NJ analysis (Figure 4.1). Species belonged to the same genus were

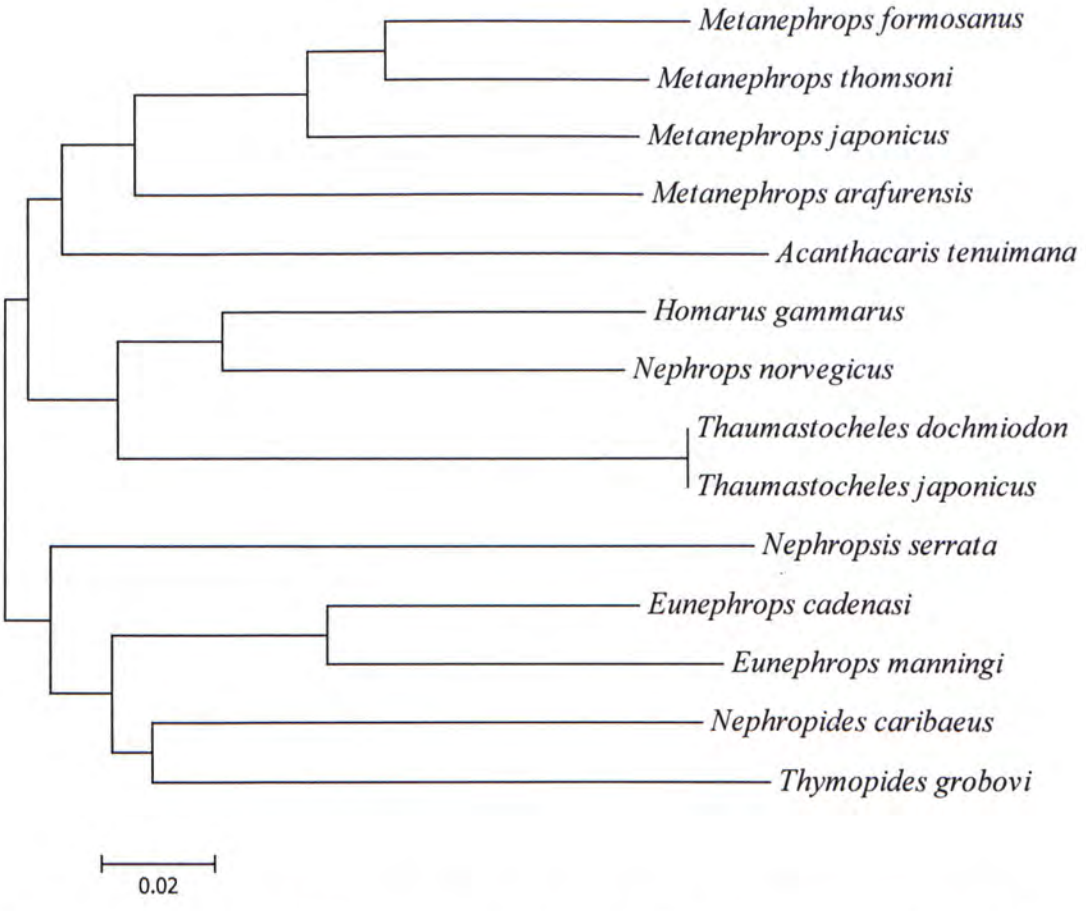

Figure 4.5 Neighbor-joining tree of mitochondrial COI gene sequences from 14 species of Nephropidae

clustered together. *Homarus gammarus* and *Nephrops norvegicus* were more related to each other than to the two *Thaumastocheles* species. In contrast to the 16S NJ analysis (Figure 4.1), COI NJ tree (Figure 4.5) showed that *Acanthacaris tenuimana* was related to the four species of *Metanephrops*. *Nephropides caribaeus* and *Thymopides grobovi* were closely related to each other and were more related to the two species of *Eunephrops* than to *Nephropsis serrata* in the NJ analysis based on COI gene sequences.

MDS analysis based on COI gene sequences (Figure 4.6) showed a similar relationship between species from Nephropidae to that of the NJ analysis inferred from COI gene sequences (Figure 4.5). In addition, results from MDS analysis (Figure 4.6) were slightly different from that of the 16S MDS analysis (Figure 4.2). In the 16S MDS analysis (Figure 4.2), the four species of *Metanephrops* were more related to each other than to other species in Nephropidae. In addition, MDS analysis based on COI gene sequences (Figure 4.6) showed that *M. arafurensis* and *M. japonicus* were closely associated to each other and were more related to *M. thomsoni* than to *M. formosanus*. Results from COI MDS analysis (Figure 4.6) were consistent to those of the COI NJ analysis (Figure 4.5) that the two species of *Eunephrops*, *Nephrops norvegicus* and *Homarus gammarus*, *Nephropides caribaeus*

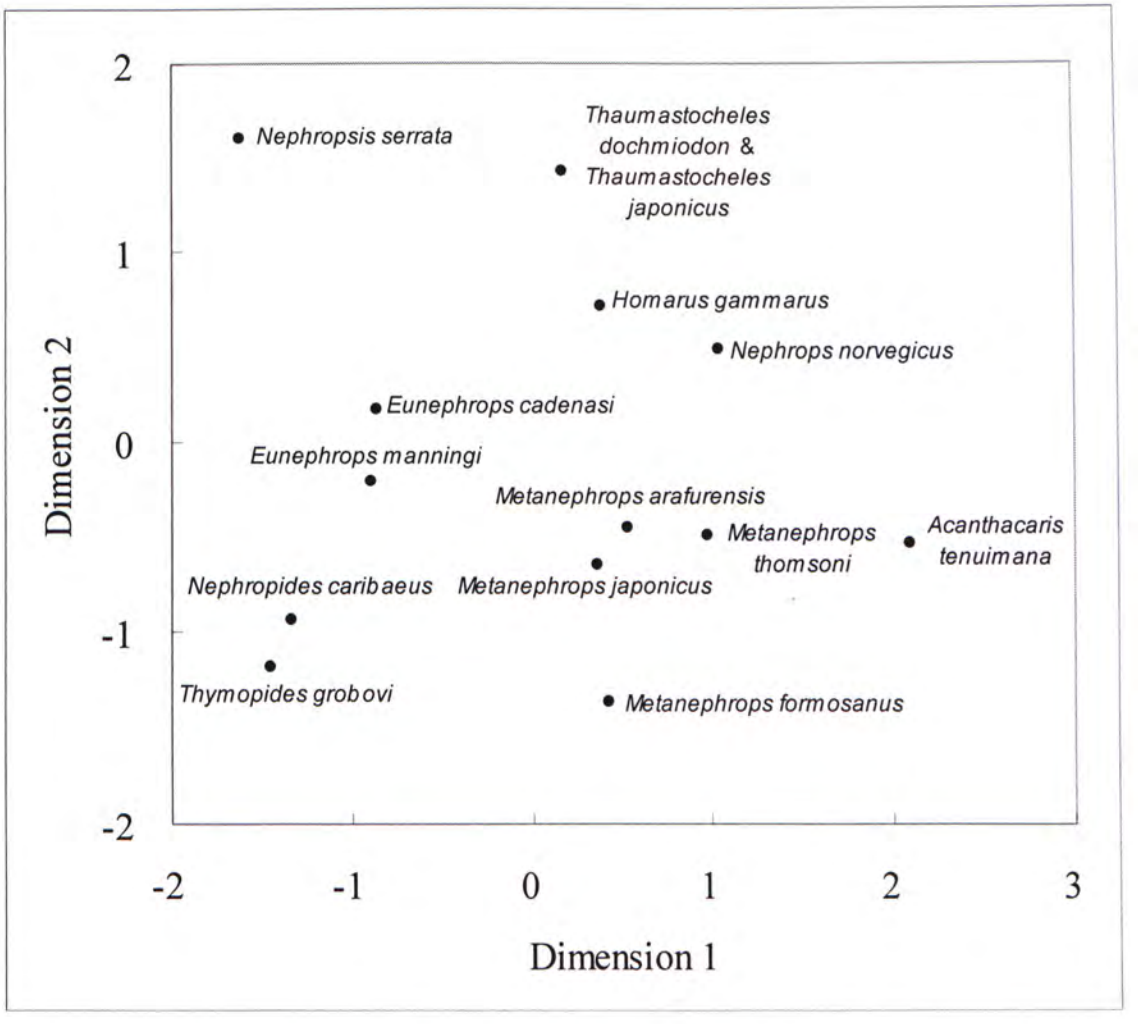

Figure 4.6 Multidimensional scaling of genetic distance based on mitochondrial COI gene sequences from 14 species of Nephropidae.

and *Thymopides grobovi* were closely related to each other, respectively. As in 16S analysis, *Nephropides serrata* was the most distantly related species to other species of Nephropidae studied in COI MDS analysis (Figure 4.6). Moreover, the two species of *Thaumastocheles* shared the same sequence and were at the same coordinates in the COI MDS analysis.

The re-run NJ analysis based on COI gene sequences (Figure 4.7) included 14 species of Nephropidae as well as the 10 test taxa. The relationships between species in Nephropidae revealed in the re-run NJ analysis (Figure 4.7) were slightly different from those in the original NJ analysis based on COI gene sequences (Figure 4.5). *Acanthacaris tenuimana* was more closely related to the test taxon *Astacus astacus* in the re-run COI NJ analysis (Figure 4.7) rather than to the four species of *Metanephrops* in the original COI NJ analysis (Figure 4.5). All conspecific individuals which were treated as test taxa were assigned to their corresponding species correctly. Similar to the re-run NJ analysis based on 16S rRNA gene sequences (Figure 4.3), the re-run COI NJ analysis (Figure 4.7) also showed that the four test taxa of *Enoplometopus* were closely related to each other. However, these four species were related to *Acanthacaris tenuimana* and *Astacus astacus* in the re-run COI NJ analysis (Figure 4.7), *Astacus astacus* was a test taxon which is

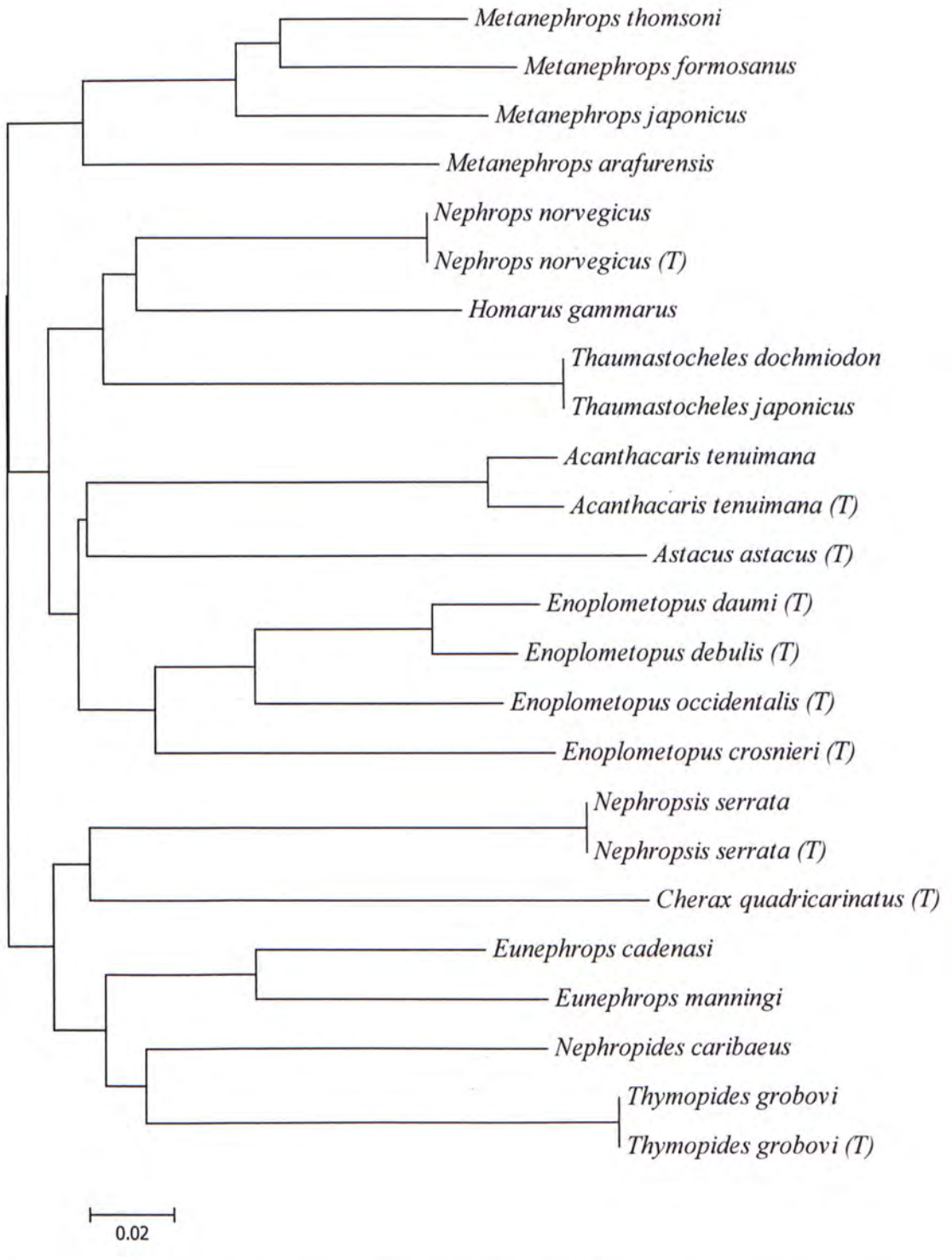

Figure 4.7 Neighbor-joining tree of mitochondrial COI gene sequences from 14 species of Nephropidae and the 10 test taxa. Species name followed by *(T)* are test taxa.

distant but relate to Nephropidae based on previous study (Crandall *et al.*, 2000). 16S re-run NJ analysis (Figure 4.3) showed that the two test taxa, *Astacus astacus* and *Cherax quadricarinatus*, were closely related to each other. The re-run NJ analysis based on COI gene sequences (Figure 4.7), however, showed that *Acanthacaris tenuimana* and *Astacus astacus*, and *Cherax quadricarinatus* and *Nephropsis serrata* were closely related to each other, respectively.

There were 14 species of Nephropidae and 10 test taxa included in the re-run MDS analysis based on COI gene sequences (Figure 4.8). The re-run MDS analysis (Figure 4.8) showed conspecific test taxa were either identical with or the most closely associated with their conspecific. In contrast to the 16S MDS re-run analysis (Figure 4.4), the COI re-run MDS analysis (Figure 4.8) showed that one of the species in *Enoplometopus* (*E. crosnieri*) was more related to another test species, *Cherax quadricarinatus* than to the remaining three species of *Enoplometopus*. Moreover, the relationship between the remaining three species of *Enoplometopus* was consistent with the result in the COI re-run NJ analysis (Figure 4.7). The three species of *Enoplometopus* were more closely associated with each other than to *Acanthacaris tenuimana*. In addition, result from COI re-run MDS analysis (Figure 4.8) was consistent with the re-run 16S NJ analysis (Figure 4.7) that the test species

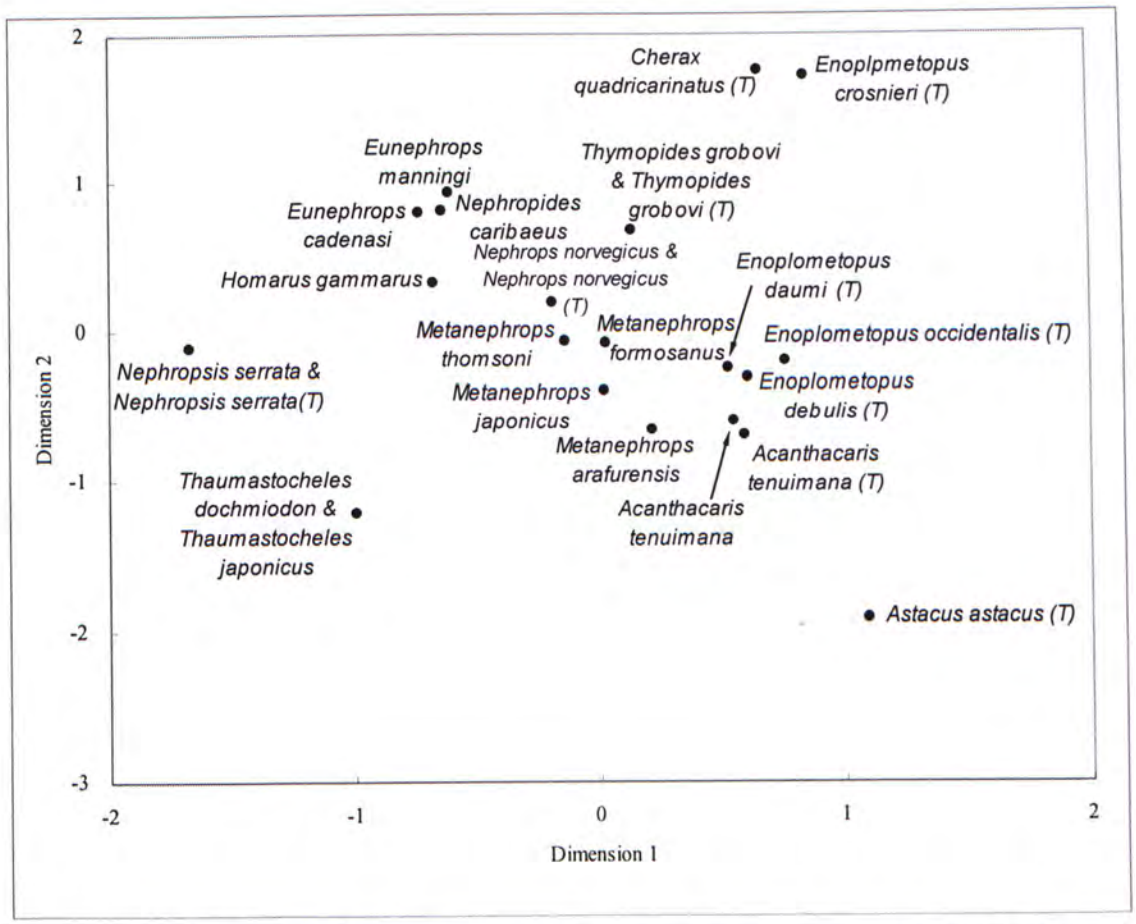

Figure 4.8 Multidimensional scaling of genetic distance based on mitochondrial COI gene sequences from 14 species of Nephropidae and the 10 test taxa. Species name followed by *(T)* are test taxa.

Astacus astacus was related to *Acanthacaris tenuimana*. However, some of the relationships among species of Nephropidae showed in the re-run MDS analysis based on COI gene sequences (Figure 4.8) were inconsistent with those in the original COI MDS analysis (Figure 4.6). For example, the original COI MDS analysis (Figure 4.6) showed that the two species of *Eunephrops* should be closely related to each other than to *Nephropides caribaeus*. In the re-run COI MDS analysis (Figure 4.8), one of the *Eunephrops* species, *E. manningi*, is closely related to *Nephropides caribaeus* than to *Eunephrops cadenasi*. The two species of *Thaumastocheles* were genetically identical. The COI re-run MDS analysis showed that these two species were related to *Nephropsis serrata* and *Homarus gammarus*. However, all other analyses (Figures 4.1 to 4.7) suggested that the two *Thaumastocheles* species were more related to *H. gammarus* or *Nephrops norvegicus*.

4.4 Discussion

In the present study, except *Thaumastocheles dochmiodon* and *T. japonicus*, each of the species from Nephropidae possesses a different 16S rRNA and COI sequence. *T. dochmiodon* was identified as *T. japonicus* before Chan and de Saint Laurent (1999) considered it to be a new species. The main morphological difference

between *T. japonicus* and *T. dochmiodon* is that the cutting teeth of the first chelae are very long and perpendicular to the fingers in the former, but are directed forward in the latter. The identical sequences in both 16S and COI genes show that the two species are genetically very similar. The taxonomic status of *T. dochmiodon* and *T. japonicus*, therefore, is put into doubt. Further study using more variable gene regions, such as nuclear genes, should be carried out to resolve whether they represent distinct species.

Three kinds of test taxa were used to test the ability of species identification at different taxonomic levels in the two profiles. The first type of test taxa consists of individuals that belong to some of the species. 16S rRNA and COI gene sequence divergence between conspecific individuals were low with a mean of 0.3 and 0.9%, respectively. The low levels of sequence divergences between conspecific individuals result in 100% success in assigning the test taxa to their corresponding species in the NJ and MDS analyses based on both genes.

The second type of test taxa is from families closely related to Nephropidae. A phylogenetic study of the reptant decapods based on three gene regions and morphology shows that family Enoplometopidae (reef clawed lobsters) is closely

related to family Nephropidae (Ahyong and O'Meally, 2004), and four species in *Enoplometopus* were chosen as the test taxa. The third type of test taxa includes species distantly related to Nephropidae. Two freshwater crayfishes, *Astacus astacus* and *Cherax quadricarinatus* were tested. 16S rRNA NJ and MDS analyses (Figures 4.3 and 4.4) showed that the test species from Enoplometopidae are more closely associated to one another than to species from Nephropidae. Moreover, the two freshwater crayfish species are more related to each other than to the other taxa in the analyses. Three groups that conform to the current classification and phylogeny, namely, clawed lobsters, reef clawed lobsters and freshwater crayfishes, are recovered in the 16S rRNA NJ analysis. The COI NJ and MDS analyses (Figures 4.7 and 4.8) also showed that test species from *Enoplometopus* are closely related to each other. However, the two freshwater crayfishes are not related to each other but are more related to certain species of Nephropidae. Moreover, only one group (reef clawed lobsters) could be recovered in the COI NJ analysis.

There are several criteria which a genetic marker needs to fulfill before it can be considered to be a good DNA barcode. One of the major criteria is that it should possess sufficient variable regions for distinguishing between different species. At the same time, it also has to contain certain conserved regions so that intraspecific

sequence divergence is low. Both 16S rRNA and COI genes in the study fulfill these two criteria because their intraspecific sequence divergence is always larger than the interspecific sequence divergence, and the two profiles set up in the present study discriminated different species and assigned those test individuals to their conspecific correctly.

It is also essential that a suitable marker should contain sufficient phylogenetic signals to assign species to major taxa, because when a sequence of an unknown taxon is added into a profile, the marker should at least contain sufficient signals to assign the unknown species to its most closely related major taxon. Three major groups (clawed lobsters, reef clawed lobsters and freshwater crayfishes) are recovered in the 16S profile in the present study. In addition, the 16S profile also showed that all congeneric species are more closely associated with each other than with species belonging to other genera. In contrast, only one group (reef lobsters) could be recovered in the COI profile in the present study, although COI profile also showed that all congeneric species are more closely related to each other than to species from other genera. In the present study, the ability of 16S profile in assigning species to major taxa is better than that of COI.

The occurrence of incorrect allocation of species to major taxa using COI barcodes in the present study may be due to the relatively small sample size (14 species studied). This indicates that there would be a chance in assigning taxa which is not belonged to Nephropidae to this family if identification solely depends on a single gene. It has been suggested that multiple DNA barcodes should be used in species identification (Stoeckle, 2003; Schander and Willassen, 2005). Therefore, species identification of Nephropidae would be more accurate when identification is based on at least two gene regions.

The present study demonstrated that barcodes of 16S rRNA and COI genes would be good candidates as species identification tool for this clawed lobster family. COI barcode is suggested to be used for species identification and the universal primers of COI gene can be used to amplify sequences from most of the animal phyla (Hebert *et al.*, 2003a). In addition, Hebert *et al.* (2003b) showed the level of sequence divergence of COI gene is able to discriminate closely related species in almost all animal phyla. COI gene is suggested to be used as one of DNA barcodes for Nephropidae. Although there are incorrect assignments of taxa to major groups based on COI gene sequences in the present study, several studies demonstrated the success of COI barcode in species identification in various groups of animals (Hebert

et al., 2003a; 2004; Hogg and Hebert, 2004; Ward *et al.*, 2005). Therefore, the universality of COI barcode can provide a common identification platform for species identification across different animal phyla.

The ability and performance of 16S rRNA and COI barcodes in identifying amphibians were compared (Vences *et al.*, 2005) and the results show that 16S rRNA gene is a better maker than COI gene in identifying amphibians as well as vertebrates. The situation observed in Vences *et al.* (2005) is the same as those in the present study. Major taxa congruent with the current classification and phylogeny can be recovered by using 16S rRNA barcode. Results from 16S barcode in the present study are generally consistent with the phylogenetic relationships as suggested in the traditional approaches (Tshudy and Babcock, 1997; Crandall *et al.*, 2000; Ahyong and O'Meally, 2004). In addition, several studies on phylogeny among and within several clawed lobster families have been based on 16S rRNA gene (e.g. Crandall *et al.*, 2000; Ahyong and O'Meally, 2004). It is expected that 16S barcode is suitable for species identification in Nephropidae.

One of the major concerns in using rRNA as DNA barcode is the difficulty in determining where to place gaps when aligning sequences with indels (Schubart *et*

al., 2000b). This problem can be overcome by analyzing unaligned rRNA gene sequences using correlation analysis based on composition vectors derived from sequence data (Chu *et al.*, 2004; 2006). The success of using such method in species discriminating in Nephropidae based on mitochondrial small subunit rRNA gene sequences implied that species discrimination in Nephropidae based on 16S rRNA is also possible by using such method.

The present study provides preliminary result that demonstrates the ability and feasibility of 16S rRNA as well as COI barcodes in species identification in family Nephropidae. Species identification of Nephropidae based on DNA barcode becomes more reliable when barcode sequences of all species are included in the profile. It is because a database does not include all species may lead to misidentification of species. Future work should be carried out to set up a comprehensive identification system for Nephropidae which include all 52 species in this family.

The property of maternal inheritance of mitochondrial DNA means that it cannot be used for identification of hybrids. Some closely related cichlids species, could hybridize extensively (Smith *et al.*, 2003). In this case, misidentification of species will be encountered if based on mitochondrial DNA barcode. Therefore,

barcode system can be improved by using nuclear gene as one of the DNA barcodes in species discrimination. Moreover, in order to obtain accurate result in identification of species, combination of multiple types of biological information should be included when making such identification.

It should be noted that there were only one to two individuals included for each species studied in the present study. This leads to a concern whether intraspecific sequence divergence is significantly and statistically larger than the interspecific sequence divergence. A recent study demonstrated that the overlap between intraspecific and interspecific genetic variability is extensive in Diptera (Meier *et al.*, 2006). Future work should be carried out to include more geographical populations of different species of Nephropidae to investigate whether a threshold value for species delimitation can be established in Nephropidae.

Chapter 5

General Conclusion

Phylogenetic relationship of *Metanephrops* based on the mitochondrial 16S rRNA and COI genes sequence analyses are elucidated in the first part of this thesis. Based on molecular data, the traditional groupings of *binghami* and *japonicus* groups are supported as they are monophyletic. The groupings of *arafurensis* and *thomsoni* groups are not supported as no monophyletic origin of these two groups is shown. Regrouping of *Metanephrops* to five groups is suggested (Table 3.10).

The present study suggests that *M. formosanus* is genetically intermediate between the members of *japonicus* group and some members of *thomsoni* group. On the other hand, results in the present study show that the *japonicus* group is closely related to some species in *thomsoni* group. This is consistent with the suggestion based on morphology that *M. formosanus* is intermediate between *japonicus* and some species in *thomsoni* groups.

Phylogenetic analyses in the present study show that *M. challengeri* and *M. neptunus* are the basal group among *Metanephrops*. *M. challengeri* is the most southern species among species in *Metanephrops*. Therefore, the present study

supports that *Metanephrops* originated near Antarctica. Most of the extant species was then dispersed and diversified in the Indo-West Pacific region from southern high attitudes. The ancestor of Atlantic group (*binghami* group) diverged from species in Indo-West Pacific at about 15.7 million years ago and migrated to the Atlantic.

Sequences of 16S rRNA and COI genes of *M. boschmai* are suggested to include with those species studied in the present study in order to construct a comprehensive phylogenetic tree of *Metanephrops*. Moreover, information from morphological data as well as molecular data should be combined to elucidate a better and more robust phylogenetic relationships and evolutionary history of *Metanephrops*.

The second part of this thesis demonstrates the ability of 16S rRNA and COI gene sequences in species discrimination for clawed lobsters in Nephropidae. Results show that both 16S rRNA and COI gene are good molecular markers in species identification for Nephropidae.

Species studied in the present study possess unique 16S rRNA and COI genes

sequences, except that *Thaumastocheles dochmiodon* and *T. japonicus* share the same 16S rRNA and COI genes sequences. Further study based on other mitochondrial or nuclear genes is suggested to resolve the taxonomic status of these two species.

The intraspecific sequence divergence of Nephropidae is much higher than that of the interspecific sequence divergence in both genes. Both gene profiles set up in the present study discriminate conspecific test species as well as other test taxa successfully. Moreover, the three major groups (clawed lobsters, reef clawed lobsters and freshwater crayfishes) are recovered in the 16S rRNA profile, while the COI profile can only recover on major group (reef clawed lobsters).

It is speculated that 16S rRNA gene sequences may be a better molecular marker in species identification for Nephropidae. COI barcode is suggested as a complement to 16S barcode for constructing a more reliable species identification system for Nephropidae.

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Appendices

Appendix 1 Aligned nucleotide sequences for a partial segment of the16S rRNA gene in *Metanephrops* used in the phylogenetic analyses. Dots indicate identity to *Metanephrops japonicus* (M. jap), dashes indicate gaps and question marks indicate missing data.

```
M. jap       GTCTGTATGG GGATTTATAA AGTCTGGCCT GCCCACTGGA ATAAAACTAA AGGGCCGCGG  [ 60]
M. and       .......... .......... .......... .........G .......... ..........  [ 60]
M. ara       .......... .......... .......... .........G .......... ..........  [ 60]
M. arm       .A........ .......... .......... .........G .......... ..........  [ 60]
M. aus       ....A..... .......... .......... .........G .......... ..........  [ 60]
M. bin       ....A....A .......... -......... .........T G....G.... ..........  [ 60]
M. cha       .......... ..T....... .......... .........T ......?... ..........  [ 60]
M. for       .......... .......... .......... .........G .......... ..........  [ 60]
M. moz       .......... .......... .......... .........G .......... ..........  [ 60]
M. nep (I)   .......... T.T....... .......... .........T .......... ..........  [ 60]
M. nep (P)   ....A..... T.T....... .......... .........T .......... ..........  [ 60]
M. rub       .........A .......... -......... .........T G....G.... ..........  [ 60]
M. sag       .......... .......... .......... .........G .......... ..........  [ 60]
M. sib       .......... .......... -......... .........G .......... ..........  [ 60]
M. sin       .......... .......... .......... .........G .......... ..........  [ 60]
M. tho (P)   .......... .......... .......... .........G .......... ..........  [ 60]
M. tho (T)   .........A .......... .......... .........G .......... ..........  [ 60]
M. vel       .......... .......... .......... .........G .......... ..........  [ 60]
A. ten       ....A....A ..T....... ......A... .........G .......... ..........  [ 60]
H. gam       ....A....A T.G....... .....AA... .........G ..C....... ..........  [ 60]

M. jap     TATTCTAACC GTGCGAAGGT AGCATAGTCA TTAGTCTTTT AATTGGAGGC TTGTATGAAG  [120]
M. and     .......... .......... .......... .......... ......G... ..........  [120]
M. ara     .......... .......... .......... .......... .....AG... ..........  [120]
M. arm     .......... .......... .......... .......... .......... ..........  [120]
M. aus     .......... .......... .......... .......... .....AG... ..........  [120]
M. bin     ....T..... .......... .......... .......... .......... .A.G.....T  [120]
M. cha     ....T..... .......... .......... .......... .....AG... .........A  [120]
M. for     .......... .......... .......... .......... .......... ..........  [120]
M. moz     .......... .......... .......... .......... ......G... ..........  [120]
M. nep (I) ....T..... .......... .......... .......... .......... ..........  [120]
M. nep (P) ....T..... .......... .......... .......... .......... ..........  [120]
M. rub     ....T..... .......... .......... .......... .....A.... ...A.....T  [120]
M. sag     .......... .......... .......... .......... ......G... ..........  [120]
M. sib     .......... .......... .......... .......... ......G... ..........  [120]
M. sin     .......... .......... .......... .......... .......... ..........  [120]
M. tho (P) .......... .......... .......... .......... .......... ..........  [120]
M. tho (T) .......... .......... .......... .......... .......... ..........  [120]
M. vel     .......... .......... .......... .......... ......G... ..........  [120]
A. ten     ....T..... .......... .......... ..T....... .......... .........T  [120]
H. gam     ....T..... .......... .......... .......C.. .......... .........T  [120]
```

```
M. jap    GGTTGAACAA GAGGCAAACT GTCTCGGAAA CAAAGTTTGA AATTAACTTT TAAGTGAAAA  [180]
M. and    .......... .......... .......... .......... .......... ..........  [180]
M. ara    ...C...... .......... .......G.. ...G...... .......... ..........  [180]
M. arm      ......... .......... .......... .......... .......... ..........  [180]
M. aus    ...C...... .......... .......G.. ...G...... .......... ..........  [180]
M. bin    ...C...... .....T.... .....A.GG. T....C.... .......... ..........  [180]
M. cha    .......... ..A....... ....T.AG.. T...A..... .T........ ..........  [180]
M. for    .......... ..A....... .......... .......... .......... ..........  [180]
M. moz    .......... .......... .......... .......... .......... ..........  [180]
M. nep (I) ......... ..A.T..... ....T.A... T...A..... .T........ ..........  [180]
M. nep (P) ......... ..A.TG.... ....T.A... T...A..... .T........ ..........  [180]
M. rub    .......... ....T..... .....A.... T....G.... .......... ..........  [180]
M. sag    .......... .......... .......... .......... .......... ..........  [180]
M. sib    .......... .......... .....AA... T..G...... .......... ..........  [180]
M. sin    .......... .....T.... .....AA... TT..A..... .......... ..........  [180]
M. tho (P) ......... .......... ........G. ...G...... .......... ..........  [180]
M. tho (T) ......... .......... ........G. .......... .......... ..........  [180]
M. vel    .......... .......... .......... .......... .......... ..........  [180]
A. ten    .....G.... ..AATT.... .....AA... T...A..... .T..G..... ..........  [180]
H. gam      .....G.... ..A....... .....AA.T. ...TAA.... .T..G..... ..........  [180]

M. jap    GGCTTAAATA TTTTAGAGGG ACGATAAGAC CCTATAAAGC TTGATAATTT T-ATATATAA  [240]
M. and    .....G.... .......... .......... .......... .......... .-....G...  [240]
M. ara    .......... .....A.... .......... .......... .......... .-........  [240]
M. arm      ......... .......... .......... .......... .......... .-..G.....  [240]
M. aus    .......... .....A.... .......... .......... .......... .-........  [240]
M. bin    .......... AC...A.... .......... .......... ..A....... .TG..AG...  [240]
M. cha    .......... .......... .......... .......... .......... .-........  [240]
M. for    .......... .......... .......... .......... .......... .-........  [240]
M. moz    .....G.... .......... .......... .......... .......... .-....G...  [240]
M. nep (I) ......... .......... .......... .......... ..A....... .-G......G  [240]
M. nep (P) ......... .G........ .......... .......... ..A....... .-T......G  [240]
M. rub      .......... CC...A.... .......... ...G...... ..A......C .-.CGAG.GG  [240]
M. sag    .....G.... .......... .......... .......... .......... .-....G...  [240]
M. sib    .......... .C........ .......... .......... .......... .-G.......  [240]
M. sin    .......... .......... .......... .......... .A........ .-G...T...  [240]
M. tho (P) .....G.... .......... .......... .......... .......... .-........  [240]
M. tho (T) .....G.... .....A.... .......... .......... .......... .-........  [240]
M. vel    .....G.... .......... .......... .......... .......... .-....G...  [240]
A. ten    .......... .....A.... .......... .........T ..A...T... A-.C.AT...  [240]
H. gam      .......... .....A.... .......... .......... ..A....... A-G.......  [240]
```

```
M. jap    A-TAAATAAG TTGTT-AGTG TTA-TATTGT TTATCTGTAA AATTATTTCG TTGGGGCGAC  [300]
M. and    .-........ .....-.... ...-...... ....T.A... .......... ..........  [300]
M. ara    G-........ .....-.... ...-...... ....A.A... .......... ..........  [300]
M. arm      .-........ .....-.... ...-...... .......... .......... ..........  [300]
M. aus    G-........ .....-...A ...-...... ....A.A... .......... ..........  [300]
M. bin    .A.......A .....-.... A..-.GC..C ...CT.ACT. .......... ......T...  [300]
M. cha    T-.......A ..A..-...A ...-....A. ....A.A.T. .......... ..........  [300]
M. for      .-........ .....-.... ...-...C.. ....T.A... .......... ..........  [300]
M. moz      .-........ .....-.... ...-...... ...CT.AC.. .......... ..........  [300]
M. nep (I) .-........ .....-...A ...-...... C...T.A.T. .......... ..........  [300]
M. nep (P) .-........ .....-...A ...-...... C...T.A.T. .......... ..........  [300]
M. rub    .-.......A .....T.... GC.A.G..AC .C.CT...T. .......... ......T...  [300]
M. sag    .-........ .....-.... ...-...... ....T.A... .......... ..........  [300]
M. sib    .-...G.... .....-...A ...-..C... C..CA.A... .......... ..........  [300]
M. sin      .-........ .....-.... ...-...CA. ....T.A... .......... ..........  [300]
M. tho (P) .-........ .....-.... ...-...CA. C...T.A... .......... ..........  [300]
M. tho (T) .-........ .....-.... ...-...CA. C...T.A... .......... ..........  [300]
M. vel    .-........ .....-.... ...-...... ....T.A... .......... ..........  [300]
A. ten    .-.......T ..A..-...A .A.-.G.CAA ...AT...T. .......... ..........  [300]
H. gam    T-..G..G.. ..AAA-...T .A.-....A. ....A.ACTG .......... ..........  [300]
```

```
M. jap      GATAATATAA TTT-GTAACT GTTTGGGGG- TTAGATTCAA ATATGTTTGT GTGTTAATGA  [360]
M. and    .......... ...-...... .....A..T- ......C... ....A..... ..T.A.....  [360]
M. ara    .......... ...-...... ....AAT.T- ..T....... ....A..... ..T.-.....  [360]
M. arm    .......... ...-...... .........- .......... .......... ..........  [360]
M. aus    .......... ...-...... ....AAT.T- ..T....... ....A..... ..T.-.....  [360]
M. bin    ..GG...... ...T...... ....TA-.T- ...A.....G GG..AC.... ACTC-.....  [360]
M. cha    .......... ...-...... .....AAAT- ..TA.....G .A..A..... T.T.-.....  [360]
M. for      .......... ...-...... ....-A..T- .......... ....A..... ..T.......  [360]
M. moz    .......... ...-...... .....A..T- ......C... ....A..... ..T.A.G...  [360]
M. nep (I) .......... ...-...... .....AAATA ..TA.....G .AG.A..... A.T.-.....  [360]
M. nep (P) .......... ...-...... .....AAATA ..TA.....G .AG.A..... ..T.-.....  [360]
M. rub      ..GG...... ...-....... ..C.AA-AT- ...A.G.... GG...C.... A.TC-G....  [360]
M. sag    .......... ...-...... .....A..T- ......C... ....A..... ..T.A.....  [360]
M. sib    .......... .C.-...... ....A.-.T- ..TA.C.... ....A..... ..T.-.....  [360]
M. sin    .......... ...-...... ....-A..T- .......... ....A..... ..T...-...  [360]
M. tho (P) .......... ...-...... ....AA.AT- .......... ....A..... ..T.......  [360]
M. tho (T) .......... ...-...... ....AA.AT- .......... ....A..... ..T.......  [360]
M. vel      .......... ...-...... .....A..T- ......C... ....A..... ..T.A.....  [360]
A. ten      .G........ ...-...... ....AAAAT- ...AT..... .A..A..... .CA.-.G...  [360]
H. gam    ...G...... ...-...... ....AAATC- .A.A..A..G .G..A..... ....-.....  [360]
```

```
M. jap   TCCAT-TAAT TGTTGATTAG AAATTTAAGT TACTTTAGGG ATAACAGCGT TATTTATTTT [420]
M. and   .....-.... .........A .......... .......... .......... .......... [420]
M. ara   .....-.... .........A .......... .......... .......... .......... [420]
M. arm   .....-.... .........A .......... .......... .......... .......... [420]
M. aus   .....-.... .........A .......... .......... .......... .......... [420]
M. bin   .....T..T. ..A......A .......... .......... .......... .......... [420]
M. cha   ...T.-.T.. .A.......A .......... .......... .......... .......... [420]
M. for     .....-.... .........A .......... .......... .......... A......... [420]
M. moz   .....-.... .........A .......... .......... .......... .......... [420]
M. nep (I) ...T.-.TG. .........A .......... .......... .......... .......... [420]
M. nep (P) ...T.-.TG. .........A .........C .......... .......... .......... [420]
M. rub   ....--.... ..G......A .......... .......... .......... .......... [420]
M. sag   .....-.... .........A .......... .......... .......... .......... [420]
M. sib   .....-.... .........A .......... .......... .......... .......... [420]
M. sin   .....-.... .........A .......... .......... .......... .......... [420]
M. tho (P) .....-.... .........A .......... .......... .......... .......... [420]
M. tho (T) .....-..G. .........A .......... .......... .......... .......... [420]
M. vel     .....-.... .........A .......... .......... .A........ .......... [420]
A. ten     ...T.---T. .A.......A ..G....... .......... .......... .......... [420]
H. gam   ...T.---G. .........A .......... .......... .......... .......... [420]
```

```
M. jap     GAGAGTTCAT ATCGATAAAA AAGTTTGCGA CCTCGATGTT GAATTAAAAT TTCTCCGTGG [480]
M. and   .......... .......... .......... .......... .......... .......... [480]
M. ara   .......... .........G .......... .......... .........T. ..T...A... [480]
M. arm   .......... .......... .......... .......... .......... .......... [480]
M. aus   .......... .........G .......... .......... .......... ..T....... [480]
M. bin   .......... .......... .......... .......... .......... .C.C...... [480]
M. cha   .......... .......... .......... .......... .......... ......A... [480]
M. for   .......... .......... .......... .......... .......... .......... [480]
M. moz   .......... .......... .......... .......... .......... .......... [480]
M. nep (I) .......... .......... .......... .......... .......... ......A... [480]
M. nep (P) .......... .......... .......... .......... .......... ......A... [480]
M. rub   .......... .......... .......... .......... .......... .C....T... [480]
M. sag   .......... .......... .......... .......... .......... .......... [480]
M. sib   .......... .........G .......... .......... .......... ..T....... [480]
M. sin   .......... .......... .......... .......... .......... ...G...C.. [480]
M. tho (P) .......... .......... .......... .......... .......... .....TA... [480]
M. tho (T) .......... .......... .......... .......... .......... ......A... [480]
M. vel     .......... .......... .......... .......... .......... .......... [480]
A. ten   .......... .....C.... .......... .......... .........A .....TA.A. [480]
H. gam   .......... .....C.... .......... .......... .........A ...G..A... [480]
```

```
M. jap      TGCAGAAGTT ACGGGGC [497]
M. and      ..T....... ......T [497]
M. ara      .....T.... ....ATA [497]
M. arm      .......... ....... [497]
M. aus      .....T.... ....ATA [497]
M. bin      .....T.... .T..A.G [497]
M. cha      .....T.... .T..A.T [497]
M. for      .....T.... ....... [497]
M. moz      ..T....... ....T.T [497]
M. nep (I)  .....T.... .T..AAT [497]
M. nep (P)  .....T.... .T..AAT [497]
M. rub      ..T..T..C. ....A.T [497]
M. sag      ..T....... ....... [497]
M. sib      .....T.... ....ATA [497]
M. sin      .....T..C. ......T [497]
M. tho (P)  .....T.... .TA...A [497]
M. tho (T)  .....T.... .T....A [497]
M. vel      ..T....... ......T [497]
A. ten      .....G.... .TA.TAG [497]
H. gam      C.T..G.... GT..A.G [497]
```

Appendix 2 Aligned nucleotide sequences for a partial segment of the COI gene in *Metanephrops* used in the phylogenetic analyses. Dots indicate identity to *Metanephrops japonicus* (M. jap), dashes indicate gaps and question marks indicate missing data.

```
M. jap      TGAGCAGGAA TAGTAGGCAC TTCTCTGAGC CTAGTAATCC GAGCCGAACT AGGTCAACCC  [  60]
M. and      .......... .......... .......... .......... .G........ .........T  [  60]
M. ara      .......... .......... ......A..T .......... ....A..... .........T  [  60]
M. arm      .......... .......... ......A... .......... .......... ..........  [  60]
M. for      ........G. .......... ......A... ..T....... ....T..G.. ...C.....T  [  60]
M. moz      .......... .......... .......... .......... .G........ .........T  [  60]
M. sag      .......... .......... .......... .......... .......... .........T  [  60]
M. sib      .......... .......T.. ...CT.A... .......... ....A..... .........T  [  60]
M. sin      .......... .......... ......A... .......... .G.....G.. .........T  [  60]
M. tho (P)  .......... .......... ......A..T .......... ........T. .........T  [  60]
M. tho (T)  .......... .......... ......A... .......... .......... .........T  [  60]
M. vel      .......... .......... ...C..A... .......... .G........ .........T  [  60]
A. ten      .....G.... ...C.A.A.. ...AT...A. T....T..T. ........T. ...C......  [  60]
H. gam      ........C. .......A.. ...AT....A ..G.....T. .T..T...T. .........A  [  60]


M. jap      GGAAGCCTTA TCGGAGACGA CCAAATCTAC AATGTAGTAG TTACTGCCCA CGCCTTCGTA  [ 120]
M. and      .....T..C. .T........ .......... ..C.....T. .A..C..... T.........  [ 120]
M. ara      .....T.... .T.....T.. ......T... ........T. .......... ..........  [ 120]
M. arm      .....T.... .......... .......... .......... ....C..... ..........  [ 120]
M. for      ..G..T..C. .T........ .......... ..C.....G. .C..C..... T.........  [ 120]
M. moz      .....T.... .......... .......... ..C.....T. .......... T.........  [ 120]
M. sag      ..G..T..C. .T........ .......... ..C.....T. .A..C..... T.........  [ 120]
M. sib      .....T.... .T.....T.. .......... ........T. .......T.. ..........  [ 120]
M. sin      .....T.... .......... .......... ..C.....T. .......... T.........  [ 120]
M. tho (P)  .....T.... .T........ .......... ..C.....T. .......... T.........  [ 120]
M. tho (T)  .....T.... .T........ .......... ........T. ....C..... T.....T...  [ 120]
M. vel      ..G..T..C. .T........ .......... ..C.....T. .A..C..... T.........  [ 120]
A. ten      ..G..TT.A. .T..C..... T.....T... ..C....... .A........ T..T..T..T  [ 120]
H. gam      ........C. .T..T..... T......... ........T. .G..C..T.. ...T..T...  [ 120]


M. jap      ATAATCTTTT TTATAGTAAT ACCTATTATA ATCGGTGGAT TTGGAAATTG ACTAGTACCC  [ 180]
M. and      .......... .......G.. ......C... .....C..G. ....T..... .T........  [ 180]
M. ara      .....T.... .C..G..T.. .......... ..T....... .......... .T.......T  [ 180]
M. arm      .......... .......G.. .......... .....C.... .......... ..........  [ 180]
M. for      .......... .C........ G..C...... .......... .......... .T........  [ 180]
M. moz      .......... .......T.. .......... .....C.... .......... ..........  [ 180]
M. sag      .......... .......G.. .......... ........G. .......... .T........  [ 180]
M. sib      .....T.... .......T.. ...C.....G ..T..C..T. .C........ .T....G...  [ 180]
M. sin      .......... .......G.. .......... ..T....... .......... .T........  [ 180]
M. tho (P)  .......... .......G.. ...C...... ........G. .......... .T....G...  [ 180]
M. tho (T)  .......... .......G.. .......... .....G.... .......... .T....G...  [ 180]
M. vel      .......... .......G.. ......C... ........G. .......... .T........  [ 180]
A. ten      .....T.... .C..G..... G..C...... .....G..C. .......C.. .T..A.T...  [ 180]
H. gam      .....T.... .C.....T.. ...C...... ..T..A..C. .C..C..C.. ...T.....A  [ 180]
```

```
M. jap      CTTATATTAG GTGCCCCAGA TATAGCTTTT CCCCGTATGA ACAATATAAG ATTCTGGCTC  [ 240]
M. and      .......... .A........ C......... ..T.....A. .T.....G.. ..........  [ 240]
M. ara      .......... .G........ ...G..C... ..T.....A. .......... ...T..A..A  [ 240]
M. arm      .......... .......... .......... ........A. .......... ..........  [ 240]
M. for      .......... .C..T..T.. .......... ........A. .......... .........T  [ 240]
M. moz      .......... .A.....G.. .........C .....C..A. .......... .........T  [ 240]
M. sag      ..A........ .G........ C......... ........A. .......... ..........  [ 240]
M. sib      ......C.C. ....T..... ......G..C ........A. .......... ......A..T  [ 240]
M. sin      ....C..... .C..A..T.. .......... ........A. ....C..... .........T  [ 240]
M. tho (P)  ..A........ .G.....C.. .......... ........A. .......... ...T.....T  [ 240]
M. tho (T)  ..A..G.... ....T..T.. .......... .....C..A. .......... ...T.....T  [ 240]
M. vel      .......... .G.....T.. C......... ..T.....A. .......G.. ..........  [ 240]
A. ten      .......... .A..A..C.. C..G..?... ..A.....A. .......... ...T......  [ 240]
H. gam      ......C... .A..T..... ......A..C ..T.....A. .......... ...T..A..G  [ 240]
```

```
M. jap      TTACCCTTTT CATTAACTCT CCTACTTACA AGAGGCATAG TAGAAAGAGG AGTGGGCACT  [ 300]
M. and      C......... .......C.. .......... .....T.... .......... ..........  [ 300]
M. ara      C.T..A..C. .......CT. A..C...... .....T.... .......... ......T..C  [ 300]
M. arm      C......... ..C....... .......... .....T.... .......... ...A......  [ 300]
M. for      C......... .GC....C.. T.....C... .....T.... .......... ...A.....C  [ 300]
M. moz      C......... .GC....C.. .......... .......... .......... .........C  [ 300]
M. sag      C......... .G.....C.. .......... .....T.... .......... ..........  [ 300]
M. sib      C.C..A..C. .CC.G..C.. G..C..C... .....T.... .......... G..C.....C  [ 300]
M. sin      C......... .CA....C.. ......C... .....T.... .......G.. ...C..T...  [ 300]
M. tho (P)  C......... .G.....C.. ...T...... .....T.... .......... G..C......  [ 300]
M. tho (T)  C......... .G.....C.. ...T...... .....T.... .......... ...C......  [ 300]
M. vel      C......... .G.....C.. .......... .....T.... .......... ..........  [ 300]
A. ten      C.T....... ..C.....T. A?.?...... .....A.... .G.....?.. T..T..A..G  [ 300]
H. gam      C.C....... .C.....AT. AT..T.A... .....A.... .......T.. ...A..A...  [ 300]
```

```
M. jap      GGATGAACTG TGTACCCACC CCTCTCGGCC GCTATCGCTC ACGCCGGTGC CTCTGTCGAC  [ 360]
M. and      .......... .A.....G.. ...T...... .......... .......... T.....T...  [ 360]
M. ara      .......... .T........ T.....A..A ........C. .T.....C.. T..A..A...  [ 360]
M. arm      .......... .A........ .......... .......... .......... ......T...  [ 360]
M. for      .......... .......... .........T .......... .......... T.....T...  [ 360]
M. moz      .......... .A........ .......... .......... .......... T.....T...  [ 360]
M. sag      .......... .A........ ...T...... .......... .......... T.....T...  [ 360]
M. sib      .......... ....T..T.. T.....A..T .......... .......C.. ...A..A...  [ 360]
M. sin      .......... .C.....G.. ......A... .........C. .......... T.....T...  [ 360]
M. tho (P)  .......... .A.....T.. ......T..G .......... .......... T.....T...  [ 360]
M. tho (T)  .......... .A.....C.. G.....T... .......... .T........ ......T...  [ 360]
M. vel      .......... .A........ ...T...... .......... .......... T.....T...  [ 360]
A. ten      ........A. .A.....C.. T..T..A..T .....T.... .?........ T..A..A..?  [ 360]
H. gam      ..G....... .C.....T.. A.....A..A ..A....... .T..T..C.. T.....T..T  [ 360]
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```
M. jap      TTAGGGATTT TCTCCCTACA CTTAGCTGGA GTCTCTTCAA TTTTAGGTGC CGTTAATTTT  [ 420]
M. and      .......... .T........ ......C..G .......... ....G..C.. ...C......  [ 420]
M. ara      .....T.... .....T.... TC.T..C..T ..T..C.... ..C....G.. A.........  [ 420]
M. arm      .....A.... .......... .......... .......... ....G..... ..........  [ 420]
M. for      .....T.... .T..G..T.. ...G..C... .......... .C........ ..........  [ 420]
M. moz      .....A.... .T........ ......C... .......... .......... ..........  [ 420]
M. sag      .......... .T........ ......C..G .......... ....G..C.. ...C..C...  [ 420]
M. sib      .....T.... .......... .........G .....C.... ..C....G.. A.........  [ 420]
M. sin      .....T..C. .T..G..T.. ......C... .......... ....G..... ..........  [ 420]
M. tho (P)  .....T.... ....G..T.. ......C... .......... .......... ..........  [ 420]
M. tho (T)  .....T.... .T..A..T.. ......C... .......... .......C.. ..........  [ 420]
M. vel      .......... .T........ ......C..G .......... ....G..C.. ...C......  [ 420]
A. ten      ....CT.... .T..A..T.. TC.G..C..T ..?..A..T. .......... T..A......  [ 420]
H. gam      .....A.... ....G..T.. TC........ ..T..A..T. ....G..... A..A......  [ 420]

M. jap      ATAACAACCG CAATCAACAT ACGAAGAAAA GGCATAACAA TAGACCGCAT ACCTTTATTT  [ 480]
M. and      .......... .......... .........G ..T....... .......A.. ..........  [ 480]
M. ara      ........T. ....T..T.. .......... .......... .......A.. ......G...  [ 480]
M. arm      .......... .......... .......... .......... .......A.. ..........  [ 480]
M. for      .....G.... .......... G........G ..T....... .......G.. ..........  [ 480]
M. moz      .......... ...C...... .........G ..T....... .......A.. ........G.  [ 480]
M. sag      .......... .......T.. .........G ..T....... .......A.. ..........  [ 480]
M. sib      ........T. .......... .........G ..G..G.... .......A.. ..........  [ 480]
M. sin      .......... .......... .......... ..T..G.... .......G.. ..........  [ 480]
M. tho (P)  .......... .......... ......G... ..T....... .......A.. ..........  [ 480]
M. tho (T)  .......... .......... ......G... ..T....... .......A.. G.........  [ 480]
M. vel      .......... .......... .........G ..T....... .......A.. ..........  [ 480]
A. ten      ........T. .T..T..... G....C..GG ..G.....T. .......G.. G..CC.?...  [ 480]
H. gam      ..G.....T. .T..T..T.. .......... ..T....... .......A.. ...C......  [ 480]

M. jap      GTTTGGTCAG TATTTATTAC CGCCGTCCTC TTGTTACTCT CTCTTCCTGT CTTAGCCGGA  [ 540]
M. and      ..G....... .......C.. .......... ..A.....T. .......... T.........  [ 540]
M. ara      ..A..A..C. ....C..C.. G...A.T..T ..AC.C..A. .A.....C.. ......A...  [ 540]
M. arm      .....A.... .......... .......... ...C...... .......... ..........  [ 540]
M. for      ..G....... .......... T...A....T ...C.G.... ....G..... ......T...  [ 540]
M. moz      ..G....... .......... .......... ..A..G.... .......... T........G  [ 540]
M. sag      ..G....... .......C.. .......... ..A.....T. .......... T.........  [ 540]
M. sib      ..G.....T. .......... A..TA....A C.AC.T.... .G.....C.. ...G..A..G  [ 540]
M. sin      ..G....... .......... ....A....T ..AC.G..T. .G..C..... ..........  [ 540]
M. tho (P)  ..G.....G. .......... .........T ..AC.G..T. .......... ......T...  [ 540]
M. tho (T)  ..G.....G. .......... .........T ..AC.G..T. .......... ......T...  [ 540]
M. vel      ..G....... .......C.. .......... ..A.....T. .......... T........G  [ 540]
A. ten      ........TC .C........ ..TTA.TT.A C.T......G .CAC...C.. ...G......  [ 540]
H. gam      ..A..A.... .......... A..A..T..T ...C....T. .C........ TC....A...  [ 540]
```

```
M. jap      GCAATTACCA TGCTACTCAC AGACCGTAAC CTAAACACCT CATTTTTTGA CCCAGCCGGA  [ 600]
M. and      .......... .......... .........T .....T..T. .......... ...G.....G  [ 600]
M. ara      ........T. .A..T..... T......... T......... .......... ...C......  [ 600]
M. arm      .......... .......... .......... ..G.....T. .......... .........G  [ 600]
M. for      ........T. ..T....... ......A..T .....T..T. .G........ ...C..T...  [ 600]
M. moz      .......... .......... T........T ........T. .......... ...C......  [ 600]
M. sag      .......... .......... .........T .....T..T. .......... .........G  [ 600]
M. sib      .......... .A..T..... T........T T....T.... .......... ...C.....T  [ 600]
M. sin      ........T. .A.....T.. ......A..T ........T. ........T. ...G..A...  [ 600]
M. tho (P)  .......... .AT....T.. ......A... .....T..T. .......C.. ...T..T...  [ 600]
M. tho (T)  ........T. .AT....T.. ......A... T....T..T. .......C.. ...T..T..G  [ 600]
M. vel      .......... .......... .......... .....T..T. .......... .........G  [ 600]
A. ten      ..T..C.... .A..TT.A.. C........T T.......A. .T........ ...C......  [ 600]
H. gam      ..T.....T. .A..TT.A.. ...T..A... T....T..T. ....C..C.. ......A..G  [ 600]
```

```
M. jap      GGTGGAGACC CTGTCCTGTA CCAACACTTA TTTTGATTCT TCGGTCACCC TGAAGTTTAC  [ 660]
M. and      .......... ....T..... T.....T... .......... ....C..... ......C..T  [ 660]
M. ara      ........T. .....T.A.. T......C.T ..C.....T. .T..C..... .........T  [ 660]
M. arm      ........T. .......... .......... .......... .T.......G .........T  [ 660]
M. for      .......... .C.....C.. .......... .......... ....C..... ......G..T  [ 660]
M. moz      ..G....... ....T..... ...G..T... .......... ....C..... ......G..T  [ 660]
M. sag      .......... ....T..... T.....T... ........T. ....A..... ......G..T  [ 660]
M. sib      ..C....... .....T.A.. T.....TC.T ..C........ .T..C..... ......A..T  [ 660]
M. sin      ..A.....T. .C.....A.. T.....T... .......... ....C..... ......G..T  [ 660]
M. tho (P)  ..C....... .C..TT.A.. .......C.G .......... ....C..... ......A..T  [ 660]
M. tho (T)  ..C..G.... .C...T.A.. .......C.G .......... ....C..... ......G..T  [ 660]
M. vel      .......... ....T..... T.....T... .......... ....C..... ......G..T  [ 660]
A. ten      ..C..G..T. .CA.T..T.. T.....TC.T G......... .T..G..... C..G..C..T  [ 660]
H. gam      ..A....... .A.....C.. T......... ..C.....T. .T..G..T.. .........T  [ 660]
```

```
M. jap      ATTCTCATTT TACCCGCCTT TGGTATAGTG TCCCATATTG TTACCCAAGA ATCTGGTAAA  [ 720]
M. and      ........C. .G..T..... ...C.....A ..T..C.... .......... ..........  [ 720]
M. ara      .....T.... ....A..... C........T ..T..C.... .A..T..... ..........  [ 720]
M. arm      .......... .......... .......... ..T....... .......... ..........  [ 720]
M. for      .....A.... ....G..T.. ...G.....A ..T....... .......... ..........  [ 720]
M. moz      .....A..C. .G..T..... ...A..G..C .......... .C........ ..........  [ 720]
M. sag      .....A..C. .G..T..... ...A.....C ..T..C.... .......... ...C......  [ 720]
M. sib      .....T...C ....G..... C..C..G..C .......... .G..A..... ..........  [ 720]
M. sin      .....T..CC ....T..... ...A...... ..T..C.... .......... ..........  [ 720]
M. tho (P)  .....T..CC ....T..... C..A.....T ..T....... .......... ..........  [ 720]
M. tho (T)  .....T..CC .G..T..... C..A.....T ..T....... .....A.... ..........  [ 720]
M. vel      .....A..C. ....T..... ...A.....C ..T..C.... .......... ..........  [ 720]
A. ten      ...T.A..C. .......T.. ...C.....C ..A..C.... .GG....... ...C..C...  [ 720]
H. gam      .....T...C .C..A..T.. .......A.T .....C.... .A..A..... ...C..G...  [ 720]
```

```
M. jap      AAAGAGGCTT TTGGCACCCT AGGTATAATT TACGCCATAC TAGCAATTGG TATTCTTGGT  [ 780]
M. and      .......... ....T..TT. .......... .......... .......... ..........  [ 780]
M. ara      .....A..A. .......TT. .......... ..T......T .......... ..........  [ 780]
M. arm      .....A.... ....T..TT. .......... .......... .......... ..........  [ 780]
M. for      .....A..A. ....T..TT. .........C .......... .......... ..........  [ 780]
M. moz      .....A..C. .......TT. .......... .......... .......... A.........  [ 780]
M. sag      ........C. ....T..TT. .......... .......... .......... ..........  [ 780]
M. sib      .....A..A. .......TT. .......... ..T....... .G........ ..........  [ 780]
M. sin      ........A. .......TT. .......... .......... .......... .........G  [ 780]
M. tho (P)  ........A. .......TT. ...C...... .....T.... .......... ..........  [ 780]
M. tho (T)  ........A. .......TT. .......... .......... .......... ..........  [ 780]
M. vel      ........A. ....T..TT. .......... ........G. .......... ..........  [ 780]
A. ten      .....AA... .......... T..G.....C .....T...A .C..T..... .G.CT.A..A  [ 780]
H. gam      ..G..A..C. ....A..T.. ...G...... ..T.....GA .......... A.........  [ 780]
```

```
M. jap      TTTGTTGTCT GAGCCCACCA TATATTTACA GTAGGAATAG ACGTTGATAC CCGAGCTTAC  [ 840]
M. and      .......... .......... C......... ..G....... .T.....C.. ...G......  [ 840]
M. ara      ..C....... .......... C.....C... .....C.... .T..G..... A.....C...  [ 840]
M. arm      .......... .......... .......... .......... .......... ..........  [ 840]
M. for      .......... ....T..... .......... ..G..C.... .T.....C.. A.....C...  [ 840]
M. moz      ........T. .G........ C......... ..G....... .T.....C.. G.....C...  [ 840]
M. sag      ........T. .......T.. C......... ..G....... .T.....C.. A.........  [ 840]
M. sib      ..C.....G. .......... C......... .....T.... .T..A..... A.....C...  [ 840]
M. sin      .......... ....T..... C........T ..G....... .T.....C.. A..G..C...  [ 840]
M. tho (P)  .......... ....T..... C......... .....T.... .T........ A.........  [ 840]
M. tho (T)  .......... ....T..... C......... .......... .T........ A.........  [ 840]
M. vel      ........T. .G.....T.. C......... .......... .T.....C.. A.........  [ 840]
A. ten      .....A..A. .......... .......... ..C..G.... .T..A..C.. .........T  [ 840]
H. gam      ..C.....T. ....A..... C........T .....T..G. .T........ A.....C...  [ 840]
```

```
M. jap      TTCACTTCTG CCACTATAAT TATTGCCGTG CCCACGGGAA TTAAAATCTT CAGGTGACTG  [ 900]
M. and      .......... .......... .......... .......... .......... ..........  [ 900]
M. ara      .....C.... .......... .........T ..T..A..T. .......... T..T...T..  [ 900]
M. arm      .......... .......... .........A .....A.... .......... .........C  [ 900]
M. for      .....C..C. .......... ......T..A .....A.... .......... ...A.....A  [ 900]
M. moz      .....C.... .......... ......T... .....A.... .......... ..........  [ 900]
M. sag      .......... .......... .......... .....A.... .......... ......G..A  [ 900]
M. sib      .....C.... .A........ ......A..T .....A.... ....G..T.. T..A...T.A  [ 900]
M. sin      .......... .......... .......... .......... .......... ..........  [ 900]
M. tho (P)  .......... .......... .........A .......... .......... ...A.....A  [ 900]
M. tho (T)  ..T....... .......... .........A .......... .......... ...A.....A  [ 900]
M. vel      .......... .......... ......T... .....A.... .......... ......G..A  [ 900]
A. ten      ..T..C.... .T........ .........? .....T.... .......T.. T.....G..T  [ 900]
H. gam      ..T....... ....A..... ......A..T ..T..A.... .......T.. ...A...T.A  [ 900]
```

```
M. jap      GGTACCCTCC AGGGCACACA AATCAACTAT AACCCCTCGC TCCTATGGGC CCTCGGCTTT  [ 960]
M. and      .......... .A..?..... .......... .......... .......A.. .........C  [ 960]
M. ara      ........A. ....T..C.. .........C ........T. .T..G..A.. ...A......  [ 960]
M. arm      .......... ....T..G.. .......... .......... .......... ..........  [ 960]
M. for      .......... .A..T..... .......... .......... .T..TA.A.. ...T......  [ 960]
M. moz      .......... .A..T..... .......... .......... .T........ ...T.....C  [ 960]
M. sag      .......... .A..T..... .......... .......... .T..G..A.. ...T.....C  [ 960]
M. sib      ..C..T.... .A..A..C.. .......... ..T.....T. ....G..... ...A..T...  [ 960]
M. sin      .......... .......... .......... .......... ....C..A.. ...T.....C  [ 960]
M. tho (P)  .......... .......... ......T... ........A. .T..C..A.. ...T..T...  [ 960]
M. tho (T)  .......... .......... .......... ........A. ....C..A.. ...T..T...  [ 960]
M. vel      .......... .A..T..... .......... .......... .T.....A.. ...T.....C  [ 960]
A. ten      ..?.....T. .......C.. .......... .G...A..T. .TT....... .T.A..A...  [ 960]
H. gam      ..C.....T. .A..T..T.. G.....T..C .GT..A..T. .T..C..A.. .T.A..T...  [ 960]

M. jap      ATTTTCCTAT TTACAGTAGG AGGCTTAACA GGAGTAGTTC TAGCCAACTC TTCCATTGAT  [1020]
M. and      .......... .......G.. .......... .......... ....?..T.. ...T......  [1020]
M. ara      .....T.... .C..T..G.. ...GC....T ..G....... .G..T..... C.........  [1020]
M. arm      .......... .......... .......... .......... .......... ...T......  [1020]
M. for      .......... .......... G...C....C .......... .......... ...T......  [1020]
M. moz      ........G. .......G.. G......... .......... ....T..T.. ...T......  [1020]
M. sag      .......... .......G.. G......... .......... ....T..T.. ..........  [1020]
M. sib      .....T.... .C..T..T.. ...AC.G..C ........G. ....T..... C..T......  [1020]
M. sin      .......... ....T..... ....C.T..C .......... ....T..T.. ..........  [1020]
M. tho (P)  .......... ....T..... ....C.C..C .....T.... ....T..T.. ...T......  [1020]
M. tho (T)  .......... ....T..... ....C.C..C .....T.... ....T..T.. ..........  [1020]
M. vel      .......... .......G.. G......... .......... ....T..T.. ..........  [1020]
A. ten      .....T?.T. .......... G..?.....? ........?T .?..A..T.. ...?.....C  [1020]
H. gam      .....T.... .......T.. T...C.C... .......... .T..T..T.. AC.T......  [1020]

M. jap      ATTATTCTCC ACGATACCTA TTACGTAGTC GCCCACTTCC ATTATGTTCT CTCTATGGGT  [1080]
M. and      .......... ....C..... ...T...... .......... .......... .........C  [1080]
M. ara      ........T. .T.....T.. C..T..G..T ..T....... .......... ......A..G  [1080]
M. arm      .......... .......... .......... ..T....... .......... ..........  [1080]
M. for      .......... ....C..... C......... ..T....... .......... .........C  [1080]
M. moz      .......... .......A.. C..T.....G .......... .......... ......A..C  [1080]
M. sag      .......... ....C..A.. C..T...... .......... ....C..... .........C  [1080]
M. sib      ........T. .T.....T.. ...T.....T .......... .C........ ......A...  [1080]
M. sin      .......... ....C..T.. C..T...... ..T....... .......... ..........  [1080]
M. tho (P)  .......... .T..C..T.. C......... ..T....... .......... ...C......  [1080]
M. tho (T)  .......... .T..C..T.. C......... ..T....... .......... ...C......  [1080]
M. vel      .......... ....C..A.. C..T...... .......... ....C..... ......A..C  [1080]
A. ten      .......... ....C..T.. C........? .....T..T. .......?T. G..G..A..A  [1080]
H. gam      ........T. ....C..A.. C..T..T..T ..T.....T. .......... A.....A..C  [1080]
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M. jap     GCTGTATTTG GTATTTTTGC CGGAGTGGCA CACTGATTCC CATTATTTAC CGGTCTTTCT  [1140]
M. and     .......... .......... T......... .......... .?C....... ......C...  [1140]
M. ara     ..C..G.... .......C.. ......A..G .......... .CC....... A..C......  [1140]
M. arm     .......... .......... .........G .......... .......... A.........  [1140]
M. for     .......... .......... T........T .......... .TC.G..C.. A.........  [1140]
M. moz     .......... .......... .......... .....G..T. .GC....... A..C......  [1140]
M. sag     .......... .......... T......... .....G..T. .G........ ......C...  [1140]
M. sib     ..C..C.... .C.....C.. .......... ........T. .CC....C.. A.....C...  [1140]
M. sin     .....G.... .......... T........C .......... .GC....... A..C......  [1140]
M. tho(P)  ........C. .......... T......... ..T....... .CC....... A..C......  [1140]
M. tho(T)  .......... .......... TA........ ..T....... .TC....... A..C......  [1140]
M. vel     .......... .......... T......... .....G..T. .GC....... ..........  [1140]
A. ten     ........C. .G........ A...A.T..T .....?..T. .GC....... T..C.....A  [1140]
H. gam     ..A..T.... .C..C..... A...A.T..C ........T. .CC....... A..C..A...  [1140]

M. jap     CTTAACCCTA AATGGTTAAA AATTCACTTC CTTACAATAT TTGCAGGAGT TAATATTACC  [1200]
M. and     .......... ....A..... .......... .......... .......... ..........  [1200]
M. ara     .......... ....AC.... .......... ..A....... ...T...T.. A.....C...  [1200]
M. arm     .......... .......... .......... .......... .......... ..........  [1200]
M. for     ........C. .G..A..... .......... ..C....... ..A....... A.....C...  [1200]
M. moz     ........C. ....AC.... .......... .......... .C........ ..........  [1200]
M. sag     ........C. ....A..... .......... .......... .......... ..........  [1200]
M. sib     .....T.... .G........ ......T... ..A..T.... .C.T...G.. A.....C...  [1200]
M. sin     .....T.... .......... .......... .....G.... .......... ......C...  [1200]
M. tho(P)  .......... ....A..... .........T .......... .......... ......C...  [1200]
M. tho(T)  .......... ....A..... .......... .......... .......G.. ......C...  [1200]
M. vel     ........C. ....A..... .......... .......... .......... ..........  [1200]
A. ten     T.A....T.. .?..AA.... ......T..T ?......... ..TTG..... A........A  [1200]
H. gam     A.A..T.... ....A..... ......T..T T.A..T.... ..A....... A.CC..C..T  [1200]

M. jap     TTTTTTCCAC AACACTTCCT A [1221]
M. and     .......... .......... . [1221]
M. ara     ..C.....T. .......... . [1221]
M. arm     ........G. .......... . [1221]
M. for     ........T. .......... G [1221]
M. moz     ........C. .......... . [1221]
M. sag     ........G. .......... . [1221]
M. sib     ..C.....C. C......... . [1221]
M. sin     ........T. .......... G [1221]
M. tho (P) ..C.....C. .......... . [1221]
M. tho (T) ..C.....C. .......... . [1221]
M. vel     ........G. .......... G [1221]
A. ten     ..C..C..G. .G.....TT. . [1221]
H. gam     ..C..C..T. ....T..... . [1221]
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Appendix 3 Aligned nucleotide sequences for a partial segment of the16S rRNA gene in Nephropidae. Dots indicate identity to *Acanthacaris tenuimana*, dashes indicate gaps and question marks indicate missing data. Species name followed by (T) are test taxa.

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Acanthacaris tenuimana          TAACCGTGCG AAGGTAGCAT AGTCATTTGT CTTTTAATTG  [ 40]
Acanthacaris tenuimana (T)      .......... .......... .......A.. ..........  [ 40]
Astacus astacus (T)             .G.......T .......... .A.....A.. ..........  [ 40]
Cherax quadricarinatus (T)      .G........ .......... .A.....A.. ..........  [ 40]
Enoplometopus crosnieri (T)     .......... .......... .A.....A.. T.........  [ 40]
Enoplometopus daumi (T)         .......... .......... .A.....A.. ..........  [ 40]
Enoplometopus debulis (T)       .......... .......... .A.....A.. ..........  [ 40]
Eunephrops cadenasi             .......... .......... .......A.. ..........  [ 40]
Eunephrops manningi             .......... .......... .......A.. ..........  [ 40]
Homarus gammarus                .......... .......... .......A.. ..C.......  [ 40]
Metanephrops arafurensis        .......... .......... .......A.. ..........  [ 40]
Metanephrops binghami           .......... .......... .......A.. ..........  [ 40]
Metanephrops formosanus         .......... .......... .......A.. ..........  [ 40]
Metanephrops japonicus          .......... .......... .......A.. ..........  [ 40]
Metanephrops thomsoni           .......... .......... .......A.. ..........  [ 40]
Nephropides caribaeus           .......... .......... .......A.. ..C.......  [ 40]
Nephrops norvegicus             .......... .......... .....C.A.. ..C.......  [ 40]
Nephrops norvegicus (T)         .......... .......... .....C.A.. ..C.......  [ 40]
Nephropsis serrata              .........A .......... .......A.G ..........  [ 40]
Nephropsis serrata (T)          .........A .......... .......A.G ..........  [ 40]
Thaumastocheles dochmiodon      .......... .......... .......A.. ..........  [ 40]
Thaumastocheles japonicus       .......... .......... .......A.. ..........  [ 40]
Thymopides grobovi              .......... .......... .A.....A.. ..........  [ 40]
Thymopides grobovi (T)          .......... .......... .A.....A.. ..........  [ 40]

Acanthacaris tenuimana          GAGGCTTGTA TGAATGGTTG GACAAGAAAT TAACTGTCTC  [ 80]
Acanthacaris tenuimana (T)      A......... .........A .......... ..........  [ 80]
Astacus astacus (T)             A.....G.A. .......... .......... A.G......T  [ 80]
Cherax quadricarinatus (T)      ......G.A. .......... ...G....GG A.G.......  [ 80]
Enoplometopus crosnieri (T)     A.AA...... .......... ...G...... AT........  [ 80]
Enoplometopus daumi (T)         A......... .......... .........C GTT.......  [ 80]
Enoplometopus debulis (T)       A......... .......... .........C GTT.......  [ 80]
Eunephrops cadenasi             .......... ....G...C. ......GGGC A........T  [ 80]
Eunephrops manningi             .......... ....G..... ......GGGC A........T  [ 80]
Homarus gammarus                .......... .......... ........GC A.........  [ 80]
Metanephrops arafurensis        AG........ ....G...C. A......GGC A.........  [ 80]
Metanephrops binghami           ......A.G. ........C. A......GGC ..........  [ 80]
Metanephrops formosanus         .......... ....G..... A.......GC A.........  [ 80]
Metanephrops japonicus          .......... ....G..... A......GGC A.........  [ 80]
Metanephrops thomsoni           .......... ....G..... A......GGC A.........  [ 80]
Nephropides caribaeus           .G........ .........A ........GC A.........  [ 80]
Nephrops norvegicus             .......... .......... ........G. A.GT......  [ 80]
Nephrops norvegicus (T)         .......... .......... ........G. A.GT......  [ 80]
Nephropsis serrata              ...T...... .......CC. ........GC ..G.......  [ 80]
Nephropsis serrata (T)          ...T...... .......CC. ........GC ..G.......  [ 80]
Thaumastocheles dochmiodon      .......... .......... ........G. A.G.......  [ 80]
Thaumastocheles japonicus       .......... .......... ........G. A.G.......  [ 80]
Thymopides grobovi              A......... .......CC. A.......GC A.........  [ 80]
Thymopides grobovi (T)          A......... .......CC. A.......GC A.........  [ 80]
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Acanthacaris tenuimana          AAAAATAAAA TTTGAATTTG ACTTTTAAGT GAAAAGGCTT [120]
Acanthacaris tenuimana (T)      .......... .......... .......... .......... [120]
Astacus astacus (T)             ...T.A...T A.....C..A .......... .......... [120]
Cherax quadricarinatus (T)      T.TCTCGG.G A........A .......... .......... [120]
Enoplometopus crosnieri (T)     GG.T.....G ......A..A .......... .......... [120]
Enoplometopus daumi (T)         T..T...... ......A..A .......... .......... [120]
Enoplometopus debulis (T)       T..T...... ......A..A .......... .......... [120]
Eunephrops cadenasi             .......G.G ......A..A .......... .......... [120]
Eunephrops manningi             ....G..G.G ......A..A .......... .......... [120]
Homarus gammarus                ...T.C..T. A......... .......... .......... [120]
Metanephrops arafurensis        GGG..C..GG ......A..A .......... .......... [120]
Metanephrops binghami           .GGG.....G C.....A..A .......... .......... [120]
Metanephrops formosanus         GG...C...G ......A..A .......... .......... [120]
Metanephrops japonicus          GG...C...G ......A..A .......... .......... [120]
Metanephrops thomsoni           GG.G.C..GG ......A..A .......... .......... [120]
Nephropides caribaeus           .........G ......A... .......... .......... [120]
Nephrops norvegicus             ..GT.C.... A......... .......... .......... [120]
Nephrops norvegicus (T)         ..GT.C.... A......... .......... .......... [120]
Nephropsis serrata              .G..G..G.G A.....A... .....CGG.. .........C [120]
Nephropsis serrata (T)          .G..G..G.G A.....A... .....CGG.. .........C [120]
Thaumastocheles dochmiodon      G..CGC.G.G G.....G... .......... .......... [120]
Thaumastocheles japonicus       G..CGC.G.G G.....G... .......... .......... [120]
Thymopides grobovi              .....A.TG. ......A... .......... .......... [120]
Thymopides grobovi (T)          .....A.TG. ......A... .......... .......... [120]

Acanthacaris tenuimana          AAATATTTTA AAGGGACGAT AAGACCCTAT AAAGTTTAAT [160]
Acanthacaris tenuimana (T)      .......... .......... .......... .......... [160]
Astacus astacus (T)             ....T..C.. .......... .......... ...AC..T.. [160]
Cherax quadricarinatus (T)      .....AG... G......... .......... ....-..T.. [160]
Enoplometopus crosnieri (T)     .....AG... G......... ........G. ...AG..T.. [160]
Enoplometopus daumi (T)         .....AA... ...T...... ........G. ...AG..T.. [160]
Enoplometopus debulis (T)       .....AA... ...T...... ........G. ...AG..T.. [160]
Eunephrops cadenasi             .......... .......... .......... ....C..G.. [160]
Eunephrops manningi             .......... .......... .......... ....C..G.. [160]
Homarus gammarus                .......... .......... .......... ....C..... [160]
Metanephrops arafurensis        .......... .......... .......... ....C..G.. [160]
Metanephrops binghami           .....AC... .......... .......... ....C..... [160]
Metanephrops formosanus         .......... G......... .......... ....C..G.. [160]
Metanephrops japonicus          .......... G......... .?........ ....C..G.. [160]
Metanephrops thomsoni           G......... G......... .......... ....C..G.. [160]
Nephropides caribaeus           .......... .......... .......... ....C..... [160]
Nephrops norvegicus             .......... .......... .......... ....C..... [160]
Nephrops norvegicus (T)         .......... .......... .......... ....C..... [160]
Nephropsis serrata              G......... .......... .......... ....C..... [160]
Nephropsis serrata (T)          G......... .......... .......... ....C..... [160]
Thaumastocheles dochmiodon      .....G.... .......... .......... ....C..... [160]
Thaumastocheles japonicus       .....G.... .......... .......... ....C..... [160]
Thymopides grobovi              .......... .......... .......... ....C..... [160]
Thymopides grobovi (T)          .......... .......... .......... ....C..... [160]
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Acanthacaris tenuimana          ATTTTA-ACA ATTAAA-TAA ATAATTTATT AGTATAATGT  [200]
Acanthacaris tenuimana (T)      ......-G.. ......-... .......... ..........  [200]
Astacus astacus (T)             ....A.---. .A...TTGCT ..T...AT.. .A----.GAG  [200]
Cherax quadricarinatus (T)      .CA.G.---. G..GGTTA.G .GTGA..TAA G..G.T.AAG  [200]
Enoplometopus crosnieri (T)     ....AT-TTG ......T.C. T.G.G..... ....A...A.  [200]
Enoplometopus daumi (T)         ..A...-.AG ......T.T. TA..A..... ....A..A.A  [200]
Enoplometopus debulis (T)       ..A...-.AG ......T.T. TA..A..G.. ....A..A.A  [200]
Eunephrops cadenasi             .A...T-..G TA...G-C.. .CT....G.. T.........  [200]
Eunephrops manningi             .A...T-... TG..G.-C.. .C........ T.........  [200]
Homarus gammarus                .A....-GT. TA...T-..G ..G.G...AA ...T....A.  [200]
Metanephrops arafurensis        .A...T-.T. TA...G-... ....G..G.. ...G.T..A.  [200]
Metanephrops binghami           .A...TTGT. .G....A... ....A..G.. ...GAT...C  [200]
Metanephrops formosanus         .A...T-.T. TA....-... ....G..G.. ...G.T..A.  [200]
Metanephrops japonicus          .A...T-.T. TA....-... ....G..G.. ...G.T..A.  [200]
Metanephrops thomsoni           .A...T-.T. TA....-... ....G..G.. ...G.T..A.  [200]
Nephropides caribaeus           .A...T-G.G CG..GG-C.. ....G..G.. ..........  [200]
Nephrops norvegicus             .A....-.T. TA...C-C.G ....A...AA ...T....A.  [200]
Nephrops norvegicus (T)         .A....-.T. TA...C-C.G ....A...AA ...T....A.  [200]
Nephropsis serrata              .A...--... ....TT-... .......... ........A.  [200]
Nephropsis serrata (T)          .A...--... ....TT-... .......... ........A.  [200]
Thaumastocheles dochmiodon      .A....-GT. TA..GC-... ..G.A....A .....T..AC  [200]
Thaumastocheles japonicus       .A....-GT. TA..GC-... ..G.A....A .....T..AC  [200]
Thymopides grobovi              .A...T-G.. CGC...-... .C..AA.G.A ..........  [200]
Thymopides grobovi (T)          .A...T-G.. CGC...-... .C..AA.G.A ..........  [200]

Acanthacaris tenuimana          CAATTAATTG TTAAATTATT TCGTTGGGGC GACG-GTAAT  [240]
Acanthacaris tenuimana (T)      T......... .......... .......... ....-.....  [240]
Astacus astacus (T)             TTTA.TT..A A----A.... .TA......T ..TA-AGG..  [240]
Cherax quadricarinatus (T)      TTTA.T..CA GC.GGG.G.. .A........ ...T-AGG..  [240]
Enoplometopus crosnieri (T)     T.T......A AAGG.A.... ..A....... ..T.-AA.GC  [240]
Enoplometopus daumi (T)         TTT...G..T .....A.... .......... ....GAG..C  [240]
Enoplometopus debulis (T)       TTT...G..T .....A.... .......... ....GAG..C  [240]
Eunephrops cadenasi             T.T....A.. .A.G...... .......... ....-A.G.C  [240]
Eunephrops manningi             T.T....A.. ..G....... .......... ....-A.G.C  [240]
Homarus gammarus                T.T...TA.A C.G....... .......... ....-A.G..  [240]
Metanephrops arafurensis        TGT...TA.A .A........ .......... ....-A....  [240]
Metanephrops binghami           TGC...C..A C......... .........T ....-AGG..  [240]
Metanephrops formosanus         .GT...T..A .A........ .......... ....-A....  [240]
Metanephrops japonicus          TGT...TC.. .A........ .......... ....-A....  [240]
Metanephrops thomsoni           ..TC..T..A .A........ .......... ....-A....  [240]
Nephropides caribaeus           T.TC...GCA .......... .......... ....-..G.C  [240]
Nephrops norvegicus             TCT...TA.A .......... .......... ....-A.G..  [240]
Nephrops norvegicus (T)         TCT...TA.A .......... .......... ....-A.G..  [240]
Nephropsis serrata              A.T..G.... GC........ .......... ....-A....  [240]
Nephropsis serrata (T)          A.T..G.... GC........ .......... ....-A....  [240]
Thaumastocheles dochmiodon      T.T...TA.A .......... .......... ..T.-A.G..  [240]
Thaumastocheles japonicus       T.T...TA.A .......... .......... ..T.-A.G..  [240]
Thymopides grobovi              T.T...-G.. .......... .......... ....-A....  [240]
Thymopides grobovi (T)          T.T...-G.. .......... .......... ....-A....  [240]
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Acanthacaris tenuimana          ATAATTT--- GTAACTGTTT AAAATTTAAT TTCAAAAATA [280]
Acanthacaris tenuimana (T)      .......--- .......... .......... .......... [280]
Astacus astacus (T)             .A...A.AAG AC......C. TTT....T.A --...T...T [280]
Cherax quadricarinatus (T)      ....-A.TAT A......... TTTG.....A .-..G.G... [280]
Enoplometopus crosnieri (T)     .....A.--- .......C.A .TT.G.A..A .-..G.T..T [280]
Enoplometopus daumi (T)         .....A.--- .......... .T..A.AT.A A-...TTT.. [280]
Enoplometopus debulis (T)       G....A.--- .......... .T.GA.AC.A A-....TT.. [280]
Eunephrops cadenasi             .......--- .........A ....A..T.A .......... [280]
Eunephrops manningi             .......--- .........A ...GA..T.A .......... [280]
Homarus gammarus                .......--- .......... ...TC.A..A .A..G.G... [280]
Metanephrops arafurensis        .......--- .......... ..TG...TGA ......T... [280]
Metanephrops binghami           .......T-- .......... T.GT-....A ....GGG... [280]
Metanephrops formosanus         .......--- .......... .GGT-...GA ......T... [280]
Metanephrops japonicus          .......--- .......... GGGGG...GA ......T..G [280]
Metanephrops thomsoni           .......--- .......... ..G.....GA ......T... [280]
Nephropides caribaeus           .......--- .......... ....A.ATTA A........G [280]
Nephrops norvegicus             .......--- .......... ...T.....A .A..G.G... [280]
Nephrops norvegicus (T)         .......--- .......... ...T.....A .A..G.G... [280]
Nephropsis serrata              .......--- .......... G.G.G..T.A .C..G.T... [280]
Nephropsis serrata (T)          .......--- .......... G.G.G..T.A .C..G.T... [280]
Thaumastocheles dochmiodon      .......--- .......... ...G...TGA .A....T... [280]
Thaumastocheles japonicus       .......--- .......... ...G...TGA .A....T... [280]
Thymopides grobovi              .......--- .......... .......T.A .A....GG.G [280]
Thymopides grobovi (T)          .......--- .......... .......T.A .A....GG.G [280]

Acanthacaris tenuimana          TTTGTGC-AT AGTGATCCTT T---TTATTG ATTAAAAGTT [320]
Acanthacaris tenuimana (T)      ......T-.. .......... .---...... .......... [320]
Astacus astacus (T)             ....A.T--A .A.....T.A A---.A.AG. .A......A. [320]
Cherax quadricarinatus (T)      .....T.--A TA......C. .---..TAG. ....G.GAA. [320]
Enoplometopus crosnieri (T)     ....-TT-T. .........A .---..T... ....T...A. [320]
Enoplometopus daumi (T)         ....ATT-.. .A......AA .---..T.-. ....T...A. [320]
Enoplometopus debulis (T)       ....ATT-.. .A......AA .---..T.-. ....C...A. [320]
Eunephrops cadenasi             .....AT-T. GA......A. .AAT.--G.. .......... [320]
Eunephrops manningi             .....AT-T. G.......A. AGGT.--G.. ....G..... [320]
Homarus gammarus                ......T-G. .A..... G---..G... .......A.. [320]
Metanephrops arafurensis        ......T-T. .A......A. .AAT.-G... .......A.. [320]
Metanephrops binghami           C....A.-TC .A......A. .TAT..GA.. .......A.. [320]
Metanephrops formosanus         ......TTT. .A......A. .AAT.-G... .......A.. [320]
Metanephrops japonicus          ......TGT. .A......A. .AAT.-G... ....G..A.. [320]
Metanephrops thomsoni           ......TTT. .A......A. .AAT.-G... .......A.. [320]
Nephropides caribaeus           ......T-T. .T......A. .AAT.--G.. .......A.. [320]
Nephrops norvegicus             ......T-G. .A..... .---...... .......A.. [320]
Nephrops norvegicus (T)         ......T-G. .A..... .---...... .......A.. [320]
Nephropsis serrata              .C...AT-T. ........G. .A-CC--GC. .......... [320]
Nephropsis serrata (T)          .C...AT-T. ........G. .A-CC--GC. .......... [320]
Thaumastocheles dochmiodon      ......T-G. .A..... G---..G... .......A.. [320]
Thaumastocheles japonicus       ......T-G. .A..... G---..G... .......A.. [320]
Thymopides grobovi              .....AT-T. .A......A. .AAT.--G.. .......A.. [320]
Thymopides grobovi (T)          .....AT-T. .A......A. .AAT.--G.. .......A.. [320]
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Acanthacaris tenuimana          TAAGTTACTT TAGGGATAAC AGCGTTATTT ATTTTGAGAG [360]
Acanthacaris tenuimana (T)      .......... .......... .......... .......... [360]
Astacus astacus (T)             .......... .......... .....A.... TC........ [360]
Cherax quadricarinatus (T)      -..A...... .......... .....A.... T....A.... [360]
Enoplometopus crosnieri (T)     .......... C......... .......... .......... [360]
Enoplometopus daumi (T)         .......... C......... .......... .......... [360]
Enoplometopus debulis (T)       .......... C......... .......... .....A.... [360]
Eunephrops cadenasi             .......... .......... .....A.... .......... [360]
Eunephrops manningi             .......... .......... .....A.... .......... [360]
Homarus gammarus                .......... .......... .......... .......... [360]
Metanephrops arafurensis        .......... .......... .......... .......... [360]
Metanephrops binghami           .......... .......... .......... .......... [360]
Metanephrops formosanus         .......... .......... .....A.... .......... [360]
Metanephrops japonicus          .......... .......... .......... .......... [360]
Metanephrops thomsoni           .......... .......... .......... .......... [360]
Nephropides caribaeus           .......... .......... .......... .......... [360]
Nephrops norvegicus             .......... .......... .......... .......... [360]
Nephrops norvegicus (T)         .......... .......... .......... .......... [360]
Nephropsis serrata              A......... .......... .......... .......... [360]
Nephropsis serrata (T)          A......... .......... .......... .......... [360]
Thaumastocheles dochmiodon      .......... .......... .......... .......... [360]
Thaumastocheles japonicus       .......... .......... .......... .......... [360]
Thymopides grobovi              .......... .......... .......... .......... [360]
Thymopides grobovi (T)          .......... .......... .......... .......... [360]

Acanthacaris tenuimana          TTCATATCGA CAAAAAAGTT TGCGACCTC- GATGTTGAAT [400]
Acanthacaris tenuimana (T)      .......... .......... .........- .......... [400]
Astacus astacus (T)             ...T...... ...G...... .........- .......... [400]
Cherax quadricarinatus (T)      ...T...... ...G.G.... .........- .......... [400]
Enoplometopus crosnieri (T)     .C........ ....G..... .........- .......... [400]
Enoplometopus daumi (T)         .......... .......... .........T .......... [400]
Enoplometopus debulis (T)       .......... .......... .........- .......... [400]
Eunephrops cadenasi             .......... .......... .........- .......... [400]
Eunephrops manningi             .......... .......... .........- ........T. [400]
Homarus gammarus                .......... .......... .........- .......... [400]
Metanephrops arafurensis        .......... T...G..... .........- .......... [400]
Metanephrops binghami           .......... T......... .........- .......... [400]
Metanephrops formosanus         .......... T......... .........- .......... [400]
Metanephrops japonicus          .......... T......... .........- .......... [400]
Metanephrops thomsoni           .......... T......... .........- .......... [400]
Nephropides caribaeus           .......... .......... .........- C......... [400]
Nephrops norvegicus             .......... .......... .........- .......... [400]
Nephrops norvegicus (T)         .......... .......... .........- .......... [400]
Nephropsis serrata              .......... ....G..... .........- .......... [400]
Nephropsis serrata (T)          .......... ....G..... .........- .......... [400]
Thaumastocheles dochmiodon      .......... .......... .........- .......... [400]
Thaumastocheles japonicus       .......... .......... .........- .......... [400]
Thymopides grobovi              .......... .......... .........- .......... [400]
Thymopides grobovi (T)          .......... .......... .........- .......... [400]
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Acanthacaris tenuimana	TAAAAATT-C TCTATAGTGC A	[421]
Acanthacaris tenuimana (T)	........-.	[421]
Astacus astacus (T)	.....G..-. .T.....A.T .	[421]
Cherax quadricarinatus (T)	........T. ...G.G...T .	[421]
Enoplometopus crosnieri (T)	.....G..-. .T....A... .	[421]
Enoplometopus daumi (T)	.....G..-. .T....A..T .	[421]
Enoplometopus debulis (T)	.....G..-. .T....A..T .	[421]
Eunephrops cadenasi	.....G..-. GT...G.C.T .	[421]
Eunephrops manningi	........-. GT..CG.C.T .	[421]
Homarus gammarus	........-. G.C..G.C.T .	[421]
Metanephrops arafurensis	....TT..-T ..C..G.... .	[421]
Metanephrops binghami	.....T.C-. C.CG.G.... .	[421]
Metanephrops formosanus	.....T..-. ..CG.G.... .	[421]
Metanephrops japonicus	.....T..-. ..CG.G.... .	[421]
Metanephrops thomsoni	.....T..-. .....G.... .	[421]
Nephropides caribaeus	........-T G.C..G.C.T .	[421]
Nephrops norvegicus	........-. G.C..G.C.T .	[421]
Nephrops norvegicus (T)	........-. G.C..G.C.T .	[421]
Nephropsis serrata	.....T..-T ATC..G.C.T .	[421]
Nephropsis serrata (T)	.....T..-T ATC..G.C.T .	[421]
Thaumastocheles dochmiodon	........-. G.C..G.C.T .	[421]
Thaumastocheles japonicus	........-. G.C..G.C.T .	[421]
Thymopides grobovi	........-. GTC..G.C.T .	[421]
Thymopides grobovi (T)	........-. GTC..G.C.T .	[421]

Appendix 4 Aligned nucleotide sequences for a partial segment of the COI gene in Nephropidae. Dots indicate identity to *Acanthacaris tenuimana*, dashes indicate gaps and question marks indicate missing data. Species name followed by (T) are test taxa.

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Acanthacaris tenuimana          GGTCAACCCG GGAGTTTAAT TGGTGACGAT CAAATTTATA  [ 40]
Acanthacaris tenuimana (T)      .......... .......... .......... ..........  [ 40]
Astacus astacus (T)             .....G..T. .A..A..... ...G..T... ..G.......  [ 40]
Cherax quadricarinatus (T)      ........A. .A..A..... C..A...... ..........  [ 40]
Enoplometopus daumi (T)         ........A. .A........ ...A...... .....C....  [ 40]
Enoplometopus debulis (T)       ........A. .A........ ...A..T... ..........  [ 40]
Enoplometopus occidentalis (T) ........A. .A........ ...A..T... ..........  [ 40]
Enoplometopus crosnieri (T)     ..C.....A. .A..AC.T.. ...A..T..C ..........  [ 40]
Eunephrops cadenasi             ..A....... .A..AC.T.. C.....T..C ........C.  [ 40]
Eunephrops manningi             ..A.....T. .A..AC.T.. ...C.....C ..G.....C.  [ 40]
Homarus gammarus                ........A. .A..CC.C.. .......... .....C..C.  [ 40]
Metanephrops arafurensis        ........T. .A...C.T.. ...A..T..C ..........  [ 40]
Metanephrops formosanus         ..C.....T. .....C.C.. ...A.....C .....C..C.  [ 40]
Metanephrops japonicus          .......... .A..CC.T.. C..A.....C .....C..C.  [ 40]
Metanephrops thomsoni           ........T. .A...C.T.. ...A.....C .....C..C.  [ 40]
Nephropides caribaeus           ..C....... .C..CC.T.. C..A.....C .....C....  [ 40]
Nephrops norvegicus             ........A. .A...C.T.. ...C..T..C ........C.  [ 40]
Nephrops norvegicus (T)         ........A. .A...C.T.. ...C..T..C ........C.  [ 40]
Nephropsis serrata              ..A....... .T.CAC.T.. C..A.....C ..........  [ 40]
Nephropsis serrata (T)          ..A....... .T.CAC.T.. C..A.....C ..........  [ 40]
Thaumastocheles dochmiodon      ........A. .TT.CC.C.. ...A..T..C .....C....  [ 40]
Thaumastocheles japonicus       ........A. .TT.CC.C.. ...A..T..C .....C....  [ 40]
Thymopides grobovi              ..C....... .T..AC.T.. ...C.....C ........C.  [ 40]
Thymopides grobovi (T)          ..C....... .T..AC.T.. ...C.....C ........C.  [ 40]

Acanthacaris tenuimana          ACGTAGTAGT AACTGCCCAC GCTTTTGTTA TAATTTTTTT  [ 80]
Acanthacaris tenuimana (T)      .......... ......G... .......... ..........  [ 80]
Astacus astacus (T)             .T........ ......T..T .......... ..........  [ 80]
Cherax quadricarinatus (T)      .T...A.C.. C..A...... ..C..C..A. .G.....C..  [ 80]
Enoplometopus daumi (T)         .T..T..... T.....A..T ..A..C.... .......C..  [ 80]
Enoplometopus debulis (T)       .T..T..... T.....A..T ..A..C.... .......C..  [ 80]
Enoplometopus occidentalis(T).T..G..... T........T ..A....... .......C..  [ 80]
Enoplometopus crosnieri (T)     ....TA.T.. T......... ..A.....A. .......C..  [ 80]
Eunephrops cadenasi             .....A.T.. ...C.....T .....C..A. ..........  [ 80]
Eunephrops manningi             .T...A.T.. G........T .....C..A. ..........  [ 80]
Homarus gammarus                .T.....T.. G..C..T... ........A. ..........  [ 80]
Metanephrops arafurensis        .T.....T.. T......... ..C..C..A. ..........  [ 80]
Metanephrops formosanus         .......G.. C..C.....T ..C..C..A. ....C.....  [ 80]
Metanephrops japonicus          .TA....... T......... ..C..C..A. ....C.....  [ 80]
Metanephrops thomsoni           .......T.. T........T ..C..C..A. ....C.....  [ 80]
Nephropides caribaeus           .T.....C.. ...C...... ..A.....A. ....C.....  [ 80]
Nephrops norvegicus             .T........ G..A..T..T .......... .......C..  [ 80]
Nephrops norvegicus (T)         .T........ G..A..T..T .......... .......C..  [ 80]
Nephropsis serrata              .T.....T.. C..A.....T .....C..A. ..........  [ 80]
Nephropsis serrata (T)          .T.....T.. C..A.....T .....C..A. ..........  [ 80]
Thaumastocheles dochmiodon      .......... C..A..T..T ..G.....A. ..........  [ 80]
Thaumastocheles japonicus       .......... C..A..T..T ..G.....A. ..........  [ 80]
Thymopides grobovi              .......T.. .........T .....C..A. .......C..  [ 80]
Thymopides grobovi (T)          .......T.. .........T .....C..A. .......C..  [ 80]
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Acanthacaris tenuimana          TATAGTAATG CCCATTATAA TTGGTGGATT TGGTAATTGG [120]
Acanthacaris tenuimana (T)      .......... ..T....... ....C..... .........A [120]
Astacus astacus (T)             ......T..A ..T....... ....C..G.. .........A [120]
Cherax quadricarinatus (T)      C.....T... ..A..C.... ....G..T.. ...G.....A [120]
Enoplometopus daumi (T)         ......T..A ..A....... .......... ...A.....A [120]
Enoplometopus debulis (T)       ......T..A ..A....... .......... ...A......  [120]
Enoplometopus occidentalis (T)......T..A ..A....... .......... .........A [120]
Enoplometopus crosnieri (T)     ......T..A .......... ....A..... ......C..A [120]
Eunephrops cadenasi             C.....T..A .......... .C..C..C.. ...A..C..A [120]
Eunephrops manningi             C.....T..A ..T....... .......C.. ...A..C..A [120]
Homarus gammarus                C.....T..A .......... ....A..C.. C..C..C..A [120]
Metanephrops arafurensis        C..G..T..A ..T....... .......... .........A [120]
Metanephrops formosanus         C......... .......... .C........ .........A [120]
Metanephrops japonicus          .........A ..T....... .C........ .........A [120]
Metanephrops thomsoni           ......G..A .......... .C.....G.. ...A.....A [120]
Nephropides caribaeus           .........A .......... .C..A..C.. ...A.....A [120]
Nephrops norvegicus             ......T..A ..T....... ....A..T.. ...A......  [120]
Nephrops norvegicus (T)         ......T..A ..T....... ....A..T.. ...A......  [120]
Nephropsis serrata              .........A ..A..C..G. ....G..... C..A..C..A [120]
Nephropsis serrata (T)          .........A ..A..C..G. ....G..... C..A..C..A [120]
Thaumastocheles dochmiodon      ......T..A ..T..C.... ....A..... C..A..C..A [120]
Thaumastocheles japonicus       ......T..A ..T..C.... ....A..... C..A..C..A [120]
Thymopides grobovi              .........A ..T.....G. ....G..C.. C..G..C... [120]
Thymopides grobovi (T)          .........A ..T.....G. ....G..C.. C..G..C... [120]

Acanthacaris tenuimana          TTAATTCCAC TCATATTAGG CGCGCCTGAT ATAGCATTTC [160]
Acanthacaris tenuimana (T)      .......... .......... T......... .......... [160]
Astacus astacus (T)             ...G....TT .A..G..... G..T...... .....T..C. [160]
Cherax quadricarinatus (T)      ...G....C. .T...C.T.. A..C...... .....C..C. [160]
Enoplometopus daumi (T)         C..G.C.... .A........ A..A...... .......... [160]
Enoplometopus debulis (T)       C..G...... .A........ A..A...... ........C. [160]
Enoplometopus occidentalis (T)...G....T. .A........ A..A..A... .......... [160]
Enoplometopus crosnieri (T)     C..G....TT .A...C.... G..C..C... .....C..C. [160]
Eunephrops cadenasi             ...G.A..T. .T...C.... A..C...... .....C..C. [160]
Eunephrops manningi             C.GG....T. .T........ A..C..A... .....C.... [160]
Homarus gammarus                C.TG.A.... .T...C.... A..T..A... ........C. [160]
Metanephrops arafurensis        ...G.A..T. .T........ G..C..A... ..G..C.... [160]
Metanephrops formosanus         ...G.A..C. .T........ ...T...... .....T.... [160]
Metanephrops japonicus          C..G.A..C. .T........ T..C..A... .....T.... [160]
Metanephrops thomsoni           ...G.G..C. .A........ G..C..C... .....T.... [160]
Nephropides caribaeus           C..G.A..T. .T........ G..A..A..C ........C. [160]
Nephrops norvegicus             ...G.....T .A........ T..T...... .....G.... [160]
Nephrops norvegicus (T)         ...G.....T .A........ T..T...... .....G.... [160]
Nephropsis serrata              C..C.A..C. .T...C.... A..C..G..C .....G..C. [160]
Nephropsis serrata (T)          C..C.A..C. .T...C.... A..C..G..C .....G..C. [160]
Thaumastocheles dochmiodon      C..G.G..T. .T........ T..C..G... .....T.... [160]
Thaumastocheles japonicus       C..G.G..T. .T........ T..C..G... .....T.... [160]
Thymopides grobovi              ..GG.A..C. .T.....G.. G.....A..C ..G..G.... [160]
Thymopides grobovi (T)          ..GG.A..C. .T.....G.. G.....A..C ..G..G.... [160]
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Acanthacaris tenuimana          CACGTATAAA TAATATAAGA TTTTGGCTTC TTCCCTTTTC [200]
Acanthacaris tenuimana (T)      ....C..... .......... .......... .......... [200]
Astacus astacus (T)             .T..C..... ...C...... .....AT.G. .C........ [200]
Cherax quadricarinatus (T)      .T..A..... .......... ..C..A...T .A..A..... [200]
Enoplometopus daumi (T)         .C..A..... .......... ..C..A..CT .A..A..C.. [200]
Enoplometopus debulis (T)       .C..A..... .......... ..C..A..CT .A..A..C.. [200]
Enoplometopus occidentalis (T).T..G..... C......... .....A...T .A..A..... [200]
Enoplometopus crosnieri (T)     .T..A..... C..C...... .....A...T .A..A..... [200]
Eunephrops cadenasi             .T..C..... .......... .....A.... ....G..C.. [200]
Eunephrops manningi             .C..C..... .......... ..C..A.... .A.....C.. [200]
Homarus gammarus                .T........ C......... .....A..G. .C........ [200]
Metanephrops arafurensis        .T........ C......... .....A..A. ....A..C.. [200]
Metanephrops formosanus         .C........ C......... ..C....... .A........ [200]
Metanephrops japonicus          .C.....G.. .......... ..C.....CT .A........ [200]
Metanephrops thomsoni           .C........ C......... .......... .A........ [200]
Nephropides caribaeus           .C..C..... ...C...... ..C..A..C. .A..A..C.. [200]
Nephrops norvegicus             .T..A..... .........G ........C. ....T..C.. [200]
Nephrops norvegicus (T)         .T..A..... .........G ........C. ....T..C.. [200]
Nephropsis serrata              .C........ C..C...... ..C.....C. .G..T..C.. [200]
Nephropsis serrata (T)          .C........ C..C...... ..C.....C. .G..T..C.. [200]
Thaumastocheles dochmiodon      .T..C..... ...C...... ......T.G. .A..T..C.. [200]
Thaumastocheles japonicus       .T..C..... ...C...... ......T.G. .A..T..C.. [200]
Thymopides grobovi              .G..C..... ...C...... ..C......T .A..A..... [200]
Thymopides grobovi (T)          .G..C..... ...C...... ..C......T .A..A..... [200]

Acanthacaris tenuimana          ACTAACTTTA TTACTTACAA GAGGAATAGT AGAAAGAGGT [240]
Acanthacaris tenuimana (T)      .......... .......... .......... G......... [240]
Astacus astacus (T)             TT........ ...T.G.TT. .G........ ...G.....A [240]
Cherax quadricarinatus (T)      T..CT.CC.T C.C....... .G........ ...G...... [240]
Enoplometopus daumi (T)         T.....A... C.TT.A..C. .T..T..... .......... [240]
Enoplometopus debulis (T)       T.....A... C..T.A..C. .T..T..... .......... [240]
Enoplometopus occidentalis (T)TT....A... .....A..T. .T........ .........C [240]
Enoplometopus crosnieri (T)     C.....AC.T .....A..T. ....T..... .......... [240]
Eunephrops cadenasi             C..T..AC.T C.C..A.... ....TC.... .......... [240]
Eunephrops manningi             G..C..GC.T C.CT.A.... ....C..... C......... [240]
Homarus gammarus                CT....A... ...T.A.... .......... ......T..A [240]
Metanephrops arafurensis        .T....C... C.C....... ....T..... .........A [240]
Metanephrops formosanus         G.....CC.T C....C.... ....T..... .........A [240]
Metanephrops japonicus          .T.....C.C C......... ....C..... .........A [240]
Metanephrops thomsoni           GT....CC.C C.T....... ....T..... .........G [240]
Nephropides caribaeus           C..C..CC.T C.C..A.... .......... .........A [240]
Nephrops norvegicus             .T....A... ...T.G.... .T........ ......T..A [240]
Nephrops norvegicus (T)         .T....A... ...T.G.... .T........ ......T..A [240]
Nephropsis serrata              ...C..GC.T ...T.A.T.. .G........ .......... [240]
Nephropsis serrata (T)          ...C..GC.T ...T.A.T.. .G........ .......... [240]
Thaumastocheles dochmiodon      CT.G...C.G C.T..A.T.. .......... .........A [240]
Thaumastocheles japonicus       CT.G...C.G C.T..A.T.. .......... .........A [240]
Thymopides grobovi              C..C..GC.. C.T..G.... ....T..... G.....C..A [240]
Thymopides grobovi (T)          C..C..GC.. C.T..G.... ....T..... G.....C..A [240]
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Acanthacaris tenuimana          GTTGGAACGG GATGAACAGT ATACCCGCCT CTTTCAGCCG [280]
Acanthacaris tenuimana (T)      .......... .......... .........C .......... [280]
Astacus astacus (T)             ..A..G..A. .......T.. T..T..C... T.AG..T.A. [280]
Cherax quadricarinatus (T)      ..C..G..A. .G..G..... T..T..T... ..AG....AT [280]
Enoplometopus daumi (T)         ..C.....C. .T.....T.. T.....T... ...G.C..T. [280]
Enoplometopus debulis (T)       ..C.....C. .T........ T.....T... ..CG.C..T. [280]
Enoplometopus occidentalis (T).........T. .T.....T.. ...T..T... ...G.T.... [280]
Enoplometopus crosnieri (T)     ........T. .T..G..... T..T..T... T.AG.T..T. [280]
Eunephrops cadenasi             .....C..T. .......... T.....T... ..C.....A. [280]
Eunephrops manningi             ..C..C..T. .C........ T..T..C..G ..C.....G. [280]
Homarus gammarus                ..A.....T. .G.....T.. C.....T..A ..C.....A. [280]
Metanephrops arafurensis        ..G..T..C. .......T.. T.....A... ..C.....A. [280]
Metanephrops formosanus         ..A..C..C. .......T.. G.....A..C ..C..G..T. [280]
Metanephrops japonicus          ..G..C..T. .......T.. G.....A..C ..C..G.... [280]
Metanephrops thomsoni           ..C..C..T. .......T.. ......T..C ..C..T..G. [280]
Nephropides caribaeus           A.C..C..A. .......... C.....C..G .........T. [280]
Nephrops norvegicus             ..A.....A. .G.....T.. T......... .....T..A. [280]
Nephrops norvegicus (T)         ..A.....A. .G.....T.. T......... .....T..A. [280]
Nephropsis serrata              ........C. .C.....T.. C..T...... ..C..T..T. [280]
Nephropsis serrata (T)          ........C. .C.....T.. C..T...... ..C..T..T. [280]
Thaumastocheles dochmiodon      .....G..A. .......T.. T.....T... ..G..T..T. [280]
Thaumastocheles japonicus       .....G..A. .......T.. T.....T... ..G..T..T. [280]
Thymopides grobovi              ..C.....C. .......... T.....C... .........T. [280]
Thymopides grobovi (T)          ..C.....C. .......... T.....C... .........T. [280]


Acanthacaris tenuimana          CTATTGCCCA TGCCGGTGCT TCAGTAGACT TAGCTATTTT [320]
Acanthacaris tenuimana (T)      .......T.. .......... .......... .......... [320]
Astacus astacus (T)             .......T.. ...A..C..A ..T....... ...GG..... [320]
Cherax quadricarinatus (T)      .A..C..... ...A..A..A .....C...C ...GC..C.. [320]
Enoplometopus daumi (T)         .A.....T.. C..A.....A .........C .G.G...... [320]
Enoplometopus debulis (T)       .A.....T.. C..A.....A .........C ...G...... [320]
Enoplometopus occidentalis (T).G.....T.. C..A.....A .........TC ...G...... [320]
Enoplometopus crosnieri (T)     .A.....T.. C..A.....A ..C.....TC ...G...... [320]
Eunephrops cadenasi             .A..C..... C..T..G..C .........C ...G...... [320]
Eunephrops manningi             .A........ ...G..G..A .........TC ...G...... [320]
Homarus gammarus                .A..C..T.. ...T..C... ..T..T..T. ...GA..... [320]
Metanephrops arafurensis        ....C..... .......C... .......... ...G...... [320]
Metanephrops formosanus         ....C..T.. C......... ..T..T.... ...G...... [320]
Metanephrops japonicus          ....C..T.. C........C ..T..C.... ...GG..... [320]
Metanephrops thomsoni           ....C..T.. C......... ..T..T.... ...G...... [320]
Nephropides caribaeus           .A..C..... C..A..A..C ..G..C.... ...G...... [320]
Nephrops norvegicus             .......T.. C......... ..T..T.... ...GA..... [320]
Nephrops norvegicus (T)         .......T.. C......... ..T..T.... ...GA..... [320]
Nephropsis serrata              ....C..... C..A..C..C ..G....... ...GC..C.. [320]
Nephropsis serrata (T)          ....C..... C..A..C..C ..G....... ...GC..C.. [320]
Thaumastocheles dochmiodon      .......T.. C.....G..C ..T..C..T. ....A..... [320]
Thaumastocheles japonicus       .......T.. C.....G..C ..T..C..T. ....A..... [320]
Thymopides grobovi              ....C..... ...T.....C ........T. ....C..C.. [320]
Thymopides grobovi (T)          ....C..... ...T.....C ........T. ....C..C.. [320]
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Acanthacaris tenuimana          TTCACTTCAT TTAGCTGGTG TTTCATCTAT TTTAGGTGCT [360]
Acanthacaris tenuimana (T)      .......... .......... .......... .......... [360]
Astacus astacus (T)             ....T.A..C ..G..A.... .A..T..G.. ......A..G [360]
Cherax quadricarinatus (T)      C..C...... ..G..C..A. .C..C..A.. .C.C..A... [360]
Enoplometopus daumi (T)         C..T.....C .....A..G. .......C.. .C.G...... [360]
Enoplometopus debulis (T)       C..T.....C .....A..A. .A.....C.. .C........ [360]
Enoplometopus occidentalis (T)...T..C... .....A..G. .C..T..A.. ...G...... [360]
Enoplometopus crosnieri (T)     ...G...... C......... .......A.. ......A..C [360]
Eunephrops cadenasi             ...G.....C C.C..C.... .A..C..C.. .C.T..A..A [360]
Eunephrops manningi             ...T.....C C.C..C..A. .G..T..C.. CC.T..G..A [360]
Homarus gammarus                C..G...... C.......A. .......... ...G.....A [360]
Metanephrops arafurensis        C..CT.A... C.T..C.... ....C..A.. .C....G..A [360]
Metanephrops formosanus         ...G.....C ..G..C..A. .C..T..A.. C........C [360]
Metanephrops japonicus          C..C..A..C ........A. .C..T..A.. .........C [360]
Metanephrops thomsoni           C..G.....C .....C..A. .C..T..A.. .........C [360]
Nephropides caribaeus           .........C .....G..G. .A..T..... ......G..G [360]
Nephrops norvegicus             ...G...... .......... .......... .........A [360]
Nephrops norvegicus (T)         ...G...... .......... .......... .........A [360]
Nephropsis serrata              C..C..C... C.G..C..C. .A..C..... CC....G... [360]
Nephropsis serrata (T)          C..C..C... C.G..C..C. .A..C..... CC....G... [360]
Thaumastocheles dochmiodon      ...G...... C.T..A..G. .G..C..... C..GA....A [360]
Thaumastocheles japonicus       ...G...... C.T..A..G. .G..C..... C..GA....A [360]
Thymopides grobovi              C..G..G..C C.T....... .G..C..... C........G [360]
Thymopides grobovi (T)          C..G..G..C C.T....... .G..C..... C........G [360]

Acanthacaris tenuimana          GTTAATTTTA TAACAACTGC TATTAATATA CGAGCAAGAG [400]
Acanthacaris tenuimana (T)      .......... .......... .......... ........G. [400]
Astacus astacus (T)             ..A....... ....T..... .........G ...AGTGT.. [400]
Cherax quadricarinatus (T)      ..A....... ....C..A.. A..C...... ...A.C.... [400]
Enoplometopus daumi (T)         ..A..C..C. .......A.. .......... ...AG..C.. [400]
Enoplometopus debulis (T)       ..A..C.... .......A.. ...C...... ...AG..CG. [400]
Enoplometopus occidentalis (T)..A..C.... .......A.. .......... ...AG..C.. [400]
Enoplometopus crosnieri (T)     ..A..C.... .......A.. A......... ...AG..C.. [400]
Eunephrops cadenasi             ..A..C..C. .G.....C.. C.....C... ...AG..A.. [400]
Eunephrops manningi             ..A..C..C. .G.....C.. ......C... ...AG..A.. [400]
Homarus gammarus                ..A....... .G........ .......... ...AG..A.. [400]
Metanephrops arafurensis        .......... .......... A......... ...AG..A.. [400]
Metanephrops formosanus         .......... ....G..C.. A..C..C..G ...AG..AG. [400]
Metanephrops japonicus          .......... .......C.. A..C..C... ...AG..A.. [400]
Metanephrops thomsoni           .......... .......C.. A..C..C... ...AGG.A.. [400]
Nephropides caribaeus           ..A..C.... .......C.. C......... ...AGG.AG. [400]
Nephrops norvegicus             ..A....... .......... .......... ...AG..A.. [400]
Nephrops norvegicus (T)         ..A....... .......... .......... ...AG..A.. [400]
Nephropsis serrata              ..A.....C. .......... C........G ..GAG..AC. [400]
Nephropsis serrata (T)          ..A.....C. .......... C........G ..GAG..AC. [400]
Thaumastocheles dochmiodon      ..A.....C. .G........ .......... ..GAG..... [400]
Thaumastocheles japonicus       ..A.....C. .G........ .......... ..GAG..... [400]
Thymopides grobovi              ..A..C.... .......C.. .......... ..TAG..A.. [400]
Thymopides grobovi (T)          ..A..C.... .......C.. .......... ..TAG..A.. [400]
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Acanthacaris tenuimana          GGATAACTAT AGACCGAATA CCATTATTTG TTTGGTCTCT  [440]
Acanthacaris tenuimana (T)      .......... .......... .......... ..........  [440]
Astacus astacus (T)             .A........ ...T...... ..TC.T.... ....A...G.  [440]
Cherax quadricarinatus (T)      .....T.C.. .......... ..C.....C. .A..A..CG.  [440]
Enoplometopus daumi (T)         .A........ .......... ..T.....C. .C..AG.AG.  [440]
Enoplometopus debulis (T)       .A........ .......... ..T.....C. .C..AG.AG.  [440]
Enoplometopus occidentalis (T).A........ .......... ..C.....C. ....AG.AG.  [440]
Enoplometopus crosnieri (T)     .A........ ......C..G .......... .C..A...G.  [440]
Eunephrops cadenasi             .A........ ......C... ..CC.T.... .C..A..AG.  [440]
Eunephrops manningi             .A........ G.....C... ..CC.T.... ....A..AG.  [440]
Homarus gammarus                .T.....A.. .......... ..C....... .A..A..AG.  [440]
Metanephrops arafurensis        .C.....A.. .......... ..T..G.... .A..A..CG.  [440]
Metanephrops formosanus         .T.....A.. ......G... ..T....... .G.....AG.  [440]
Metanephrops japonicus          .C.....A.. ......C... ..T....... .......AG.  [440]
Metanephrops thomsoni           .T.....A.. .......... ..T....... .G.....GG.  [440]
Nephropides caribaeus           .C.....A.. ......G..G ...C.T..C. .C..A..CG.  [440]
Nephrops norvegicus             .A.....A.. ......T... .......... .A..A..AG.  [440]
Nephrops norvegicus (T)         .A.....A.. ......T... .......... .A..A..AG.  [440]
Nephropsis serrata              .A...T.... .......... ..TC.C.... .C.....AG.  [440]
Nephropsis serrata (T)          .A...T.... .......... ..TC.C.... .C.....AG.  [440]
Thaumastocheles dochmiodon      .A.....AT. .......... ..T.....C. .......AG.  [440]
Thaumastocheles japonicus       .A.....AT. .......... ..T.....C. .......AG.  [440]
Thymopides grobovi              .C.....A.. ......C..G ...C.T.... .C..A..AG.  [440]
Thymopides grobovi (T)          .C.....A.. ......C..G ...C.T.... .C..A..AG.  [440]


Acanthacaris tenuimana          TTTCATTACC GTTATCTTGC TTTTACTCGC CCTTCCAGTT  [480]
Acanthacaris tenuimana (T)      ...T...... ........A. .......T.. T........C  [480]
Astacus astacus (T)             A..T.....A .CAG.TC.CT .AC..T.AT. TT.A..T..A  [480]
Cherax quadricarinatus (T)      C........T .CAG.AC.T. .GC.TT.AT. ...C..C...  [480]
Enoplometopus daumi (T)         G..T.....T .CCG....AT .AC....AT. A........C  [480]
Enoplometopus debulis (T)       A..T...... .CCG..C.A. .A.....AT. A..C.....C  [480]
Enoplometopus occidentalis (T)A..T...... .CA..T..A. .AC.C..TT. A.........  [480]
Enoplometopus crosnieri (T)     A..T...... .C......AT .A......T. A..A..T..A  [480]
Eunephrops cadenasi             C..T...... .CCG..C... .CC.T..TT. ...A..C...  [480]
Eunephrops manningi             ...T...... .C.G.AC.T. ..C.C..GT. G..C..C...  [480]
Homarus gammarus                A..T.....A .CAG.TC.TT .GC....TT. ......T...  [480]
Metanephrops arafurensis        A.....C..G .CC..TC.TT .AC.C..AT. A.....C..C  [480]
Metanephrops formosanus         A..T.....T .CC...C.TT .GC.G...T. T..G..T..C  [480]
Metanephrops japonicus          A..T...... .CCG..C.CT .G......T. T.....T..C  [480]
Metanephrops thomsoni           A..T...... .CCG..C.TT .AC.G..TT. T.....T..C  [480]
Nephropides caribaeus           ......C..A .C.G.AC..T .AC.T...T. .T.A......  [480]
Nephrops norvegicus             G..T.....A .CAG.AC.TT .A.....TT. G.....C...  [480]
Nephrops norvegicus (T)         G..T.....A .CAG.AC.TT .A.....TT. G.....C...  [480]
Nephropsis serrata              C..T...... .CCG.T..A. .A...T.AT. ...G..T..C  [480]
Nephropsis serrata (T)          C..T...... .CCG.T..A. .A...T.AT. ...G..T..C  [480]
Thaumastocheles dochmiodon      A..T.....A .CAG.A..AT .A..G...T. T.....C...  [480]
Thaumastocheles japonicus       A..T.....A .CAG.A..AT .A..G...T. T.....C...  [480]
Thymopides grobovi              ...T..C..T .CCG.TC.A. .GC....... TT.G......  [480]
Thymopides grobovi (T)          ...T..C..T .CCG.TC.A. .GC....... TT.G......  [480]
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Acanthacaris tenuimana          TTAGCCGGAG CTATTACTAT ACTTTTAACT GACCGTAATT [520]
Acanthacaris tenuimana (T)      .......... .......... .......... ..T..C.... [520]
Astacus astacus (T)             ..G..A..T. .......... .T.G.....A ..T....... [520]
Cherax quadricarinatus (T)      C....A.... .A........ ....C.T..A .....A...C [520]
Enoplometopus daumi (T)         C....T.... .A........ ...CC..... ..T..A.... [520]
Enoplometopus debulis (T)       .......... .A........ ...C...... ..T..A.... [520]
Enoplometopus occidentalis (T).....T..C. .A........ G......... ..T..A.... [520]
Enoplometopus crosnieri (T)     .....T.... .A........ G........C .....A...C [520]
Eunephrops cadenasi             C....T.... .A..C..... ...C.....A ........CC [520]
Eunephrops manningi             C....T.... .A........ ...CC....A ........CC [520]
Homarus gammarus                C....A.... .......... .........A ..T..A..C. [520]
Metanephrops arafurensis        .....A.... .A........ ....C.C... ........C. [520]
Metanephrops formosanus         .....T.... .A........ GT.AC.C..A .....A...C [520]
Metanephrops japonicus          .......... .A.....C.. G..AC.C..A ........CC [520]
Metanephrops thomsoni           .....T.... .A.....C.. .T.AC.T..A .....A..CC [520]
Nephropides caribaeus           .......... .A..C..... ...A.....A .....C..CC [520]
Nephrops norvegicus             .....A.... .A........ ...A.....A ..T..A.... [520]
Nephrops norvegicus (T)         .....A.... .A........ ...A.....A ..T..A.... [520]
Nephropsis serrata              C.G..G.... .......A.. .........A .....A..CC [520]
Nephropsis serrata (T)          C.G..G.... .......A.. .........A .....A..CC [520]
Thaumastocheles dochmiodon      .....A..G. ....C..A.. .........A .....A..CC [520]
Thaumastocheles japonicus       .....A..G. ....C..A.. .........A .....A..CC [520]
Thymopides grobovi              .....T..G. .A..C..C.. ...CC.G..A .....C...C [520]
Thymopides grobovi (T)          .....T..G. .A..C..C.. ...CC.G..A .....C...C [520]

Acanthacaris tenuimana          TAAATACCTC TTTTTTTGAC CCCGCCGGGG GCGGGGATCC [560]
Acanthacaris tenuimana (T)      .......... .......... .......... .......C.. [560]
Astacus astacus (T)             .......T.. A......... ..T.....A. .T..T..C.. [560]
Cherax quadricarinatus (T)      ....C..TA. ...C...... ..T..T..C. .G..A..... [560]
Enoplometopus daumi (T)         ....C..... A..C...... ..A..T..C. .A..T..C.. [560]
Enoplometopus debulis (T)       .......... A..C...... ..A..T..C. .A..T..C.. [560]
Enoplometopus occidentalis (T).......T.. A......... ........T. .A..T..C.. [560]
Enoplometopus crosnieri (T)     .C.....T.. C..C.....T ..T..T..A. .T..A..... [560]
Eunephrops cadenasi             .......T.. C......... ........A. .A.....C.. [560]
Eunephrops manningi             ....C..T.. C.....C... ..T..G..A. .......C.. [560]
Homarus gammarus                .......T.. A..C..C... ..A..A.... .A..A..C.. [560]
Metanephrops arafurensis        ....C..... A......... ........A. .T..A..... [560]
Metanephrops formosanus         .......T.. G......... .....T..A. .T..A..C.. [560]
Metanephrops japonicus          ....C..... A......... ..A.....A. .T..A..C.. [560]
Metanephrops thomsoni           .......T.. A.....C... ..T..T..A. ....A..C.. [560]
Nephropides caribaeus           .......... C......... .....G..A. ....A..C.. [560]
Nephrops norvegicus             .......T.. G......... ..A..A..A. .A..A..C.. [560]
Nephrops norvegicus (T)         .......T.. G......... ..A..A..A. .A..A..C.. [560]
Nephropsis serrata              .......T.. C..C...... .....A..C. .G..A..C.. [560]
Nephropsis serrata (T)          .......T.. C..C...... .....A..C. .G..A..C.. [560]
Thaumastocheles dochmiodon      .......T.. .......... ..G..A..A. .A..A..C.. [560]
Thaumastocheles japonicus       .......T.. .......... ..G..A..A. .A..A..C.. [560]
Thymopides grobovi              ....C..T.. C.....C... ..A....... .A..A..C.. [560]
Thymopides grobovi (T)          ....C..T.. C.....C... ..A....... .A..A..C.. [560]
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Acanthacaris tenuimana	CATTCTTT	[568]
Acanthacaris tenuimana (T)	.....	[568]
Astacus astacus (T)	A...T.A.	[568]
Cherax quadricarinatus (T)	T.....C.	[568]
Enoplometopus daumi (T)	T...T.A.	[568]
Enoplometopus debulis (T)	T...T.A.	[568]
Enoplometopus occidentalis(T)	T.....	[568]
Enoplometopus crosnieri (T)	AG.C....	[568]
Eunephrops cadenasi	.....	[568]
Eunephrops manningi	.....C.	[568]
Homarus gammarus	AG....C.	[568]
Metanephrops arafurensis	TG.CT.A.	[568]
Metanephrops formosanus	.G.C..C.	[568]
Metanephrops japonicus	TG.C..G.	[568]
Metanephrops thomsoni	.G..T.A.	[568]
Nephropides caribaeus	AG....C.	[568]
Nephrops norvegicus	AG.A....	[568]
Nephrops norvegicus (T)	AG.A....	[568]
Nephropsis serrata	T.....	[568]
Nephropsis serrata (T)	T.....	[568]
Thaumastocheles dochmiodon	TG.G..G.	[568]
Thaumastocheles japonicus	TG.G..G.	[568]
Thymopides grobovi	AG.C..C.	[568]
Thymopides grobovi (T)	AG.C..C.	[568]